THE AMINO ACID POOL IN *ESCHERICHIA COLI*

ROY J. BRITTEN AND F. T. McCLURE

Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C., and Applied Physics Laboratory, The Johns Hopkins University, Silver Spring, Maryland

I. INTRODUCTION

"For an angel went down at a certain season into the pool and troubled the water."

John 5:4

Bacteria maintain internally synthesized small molecules at high internal concentrations and in addition have the capacity to concentrate many compounds from the environment. Since the majority of these compounds are intermediates in synthesis, they are collectively termed the pool of metabolic intermediates or, simply, the "pool." However, the state of organization and ultimate chemical fate of exogenous compounds concentrated by the cell may be different from those of identical compounds synthesized by the cell.

Since the mechanism by which high internal concentrations are maintained is not understood and the processes are obviously complex, it appears fruitless to enter into an extended discussion of the meaning of the term "pool." Therefore, we will simply define the "pool" as the total quantity of low molecular weight compounds that may be extracted from the cell under conditions such that the macromolecules are not degraded into low molecular weight subunits, for example, brief exposure to 5% trichloroacetic acid at room temperature.

Experiments have in general been designed to answer the following three questions: What are the mechanisms by which exogenous compounds are concentrated? What states of organization exist for compounds in the pool? What is the relationship of the pool to the mechanism of macromolecular synthesis?

At the present time unequivocal answers do

not exist for any of these three questions. However, the large body of experimental evidence does provide a restrictive set of conditions which theoretical models must satisfy, and supplies a background for the formulation of more refined questions.

The present paper is a description of several years of experimental work which in general has only been briefly described in print (2–4, 6). The implications of the evidence for the mechanism of pool formation are discussed in relation to several possible models. A mathematical analysis of the implications of the models is given in an appendix.

II. Principal Features of Pool Formation and Maintenance

A. Introductory Discussion

Pool formation is an expression of the ability of the cell to obtain nutrients present at very low concentrations in the environment and to supply them to the synthetic machinery at high concentrations. This, perhaps, allows significant simplification of subsequent steps in macromolecular synthesis. One of the principal questions is whether the internally concentrated substances are free in solution within the cell or held in a more complex fashion. If the pool is simply a concentrated solution that pervades the cell, then the synthetically active structures within the cell are bathed in this solution, which is thus the "medium" in which synthesis occurs. On the other hand, the amino acids of the pool may be more closely associated with the substructures of the cell responsible for protein synthesis. They simply might be trapped in such substructures or they might be bound to them by labile chemical bonds. In the latter case it would be highly important to know the nature of the binding sites and how intimately they are related to the synthetic activities.

Since there is a large body of experimental evidence presented in this section, it seems well, for purposes of clarity, to summarize in advance the principal features of the pool that have been demonstrated:

1) Passage through the pool appears to be an obligate step for incorporation of an exogenous amino acid into protein.

2) Amino acids present in the pool are incorporated into protein at random, regardless of the length of time they have been in the pool.

3) Peptides do not appear to be intermediates in protein synthesis.

4) An energy source (such as glucose) is required for pool formation to occur at normal rates but is not required for maintenance of the pool for relatively long periods.

5) Specific pool formation mechanisms exist for each amino acid or group of structurally similar amino acids.

6) For any given amino acid there appears to be maximum pool size (or saturation value) at large external concentrations.

7) Any damage to the cells' integrity, or even mild treatments (for a bacterial cell), such as osmotic shock, leads to loss of the pool.

8) Exchange between pool and external amino acids occurs at a high rate, not only when there is steady flow through the pool but also in absence of glucose or at 0 C when the flow through the pool is strongly suppressed (conditions which also suppress pool formation).

B. A Typical Experiment

The experiments were performed with uniformly C^{14}-labeled amino acids of very high specific radioactivity, chromatographically prepared from *Chlorella* protein hydrolyzates (14, p. 47). The amino acids were chromatographically pure, and their purity was further checked by the suppression of the incorporation by *Escherichia coli* of a given labeled amino acid when pure amino acid carrier was present.

For low amino acid concentrations, constant pool sizes are established within 1 min. As a result, a technique had to be developed for sampling at 5- to 10-sec intervals in order to measure the kinetics of pool formation (6).

In a typical experiment, a suspension of *E. coli* strain B (ATCC 11303) was aerated in an open beaker in a temperature-controlled water bath. At the start of the experiment, the tracer was injected with a hypodermic syringe, in a moderate volume of medium, to give instantaneous mixing. Samples were withdrawn with a hypodermic syringe fitted with a stop to deliver a reproducible volume. These samples were either immediately filtered on a collodion membrane or injected into an equal volume of 10% trichloroacetic acid. The radioactivity of the cells collected on the filter measured the total incorporation, that is, the sum of the amount of labeled amino acid in the pool and the amount incorporated into protein. After about 10 min, the sample that was diluted into trichloroacetic acid was filtered on a similar collodion membrane. As the

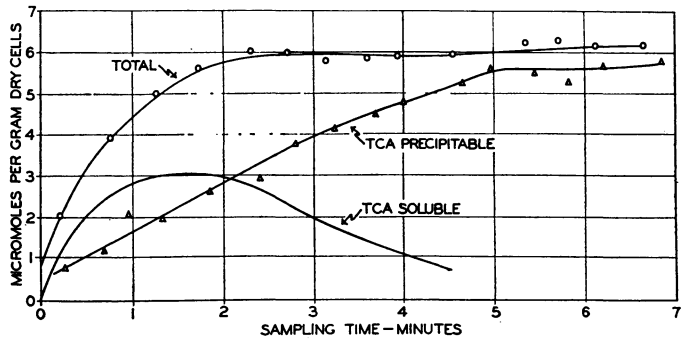

FIG. 1. *Incorporation of C^{14}-proline by a suspension of growing E. coli cells. The temperature was 24 C and the generation time, about 2½ hr. The suspension contained glucose, ammonia, mineral salts, L (—) C^{14}-proline at 1.2 × 10^{-6} M, and 0.2 mg, dry weight, of cells per ml (equal to 0.8 mg, wet weight, per ml). TCA = trichloroacetic acid.*

pool was extracted by the 5% trichloroacetic acid, the radioactivity of this filter measured the incorporation into macromolecules. Most of the amino acids are utilized only for protein synthesis. For these amino acids, the radioactivity of the trichloroacetic acid precipitate is a direct measure of the incorporation into protein.

To avoid curling, the wet filters were cemented to plastic planchettes with rubber cement. The very thin, flat, and uniform layers of cells on the collodion membranes gave precisely reproducible counting rates. Thus the pool radioactivity could be accurately calculated by subtracting the quantity incorporated into protein from the total.

Figure 1 shows the results of an experiment measuring the uptake of C^{14}-proline. After a lag of less than 10 sec, the proline is taken up into the compounds of the trichloroacetic acid precipitate at a constant rate until the supply in the medium approaches exhaustion. The total quantity taken up into the cell rises rapidly at first and then parallels the uptake into the protein, until the external amino acid is almost exhausted. The difference between these two curves measures the quantity of proline in the pool. This quantity rises rapidly at first, then remains constant for a period, and finally decreases as the proline is transferred to the protein after the supply in the medium is exhausted. The maximum concentration of trichloroacetic acid-soluble proline per milliliter of cells is 600 times the initial concentration of proline in the medium.

Before the supplemental proline is added, and after it is exhausted, the cell internally synthesizes proline as required for protein. In this experiment the supplemental proline supplies, at maximum, half of this requirement, while at higher concentrations internal synthesis is almost completely suppressed (see Appendix). Such a suppression, however, does not occur for all amino acid supplements (14, p. 196).

The radioactive material extracted from the cells with trichloroacetic acid after a 1-min exposure to C^{14}-proline, in a similar experiment, has been shown to be authentic proline by paper chromatographic fingerprinting (14, p. 191). When relatively high concentrations (10^{-4} M) of proline are supplied, however, the cells convert some of the proline to arginine and glutamic acid after a delay of about ½ hr. In such an experiment, the rate of incorporation of radioactivity into protein increases appreciably after ½ hr, and chromatography shows the presence of the other amino acids in the pool. This is an interesting example of the induced reversal of reaction sequences which are normally entirely unidirectional.

The experiments with C^{14}-proline described below have been performed under conditions in which conversion to other amino acids is negligible. For experiments with other amino acids, such as valine, which is rapidly converted to leucine, chromatography has been used to check the results, and proper allowance has been made for conversions or degradations occurring in the pool.

C. Passage through Pool Obligate for Entry into Protein

The experiment illustrated in Fig. 1 shows that the amino acids of the pool are readily availa-

ble for protein synthesis. It is not immediately clear, however, that entry into the pool is a necessary step in protein synthesis. Two possible interpretations are shown in Fig. 2. The lower diagram shows the incorporation curve to be expected if the externally added labeled amino acid must mix with a pre-existing unlabeled pool before entering the protein. The rate of entry of radioactivity into protein is initially zero.

On the other hand, if the labeled amino acid by-passes the pool, it will initially enter the protein at a rate determined by the relative by-pass flow. The upper diagram on Fig. 2 is for an extreme case in which the by-pass flow is large and the quantity of amino acid pre-existing in the pool is so great that the specific radioactivity of the added amino acid is reduced significantly by dilution. Examples of large by-pass flows occur in the incorporation of nucleic acid bases into ribonucleic acid (11).

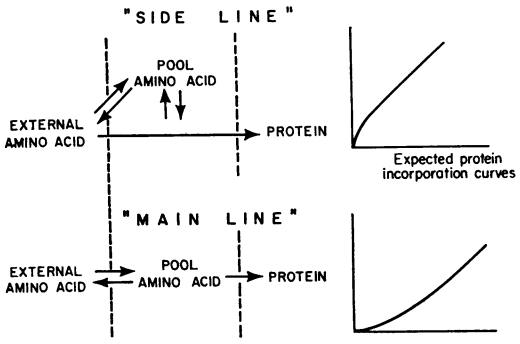

FIG. 2. *Schematic illustration of two interpretations of the function of the pool. At the right are shown the expected curves for incorporation of C^{14}-proline into the protein when the cells have been pretreated with C^{12}-proline.*

In order to assess the possible existence of a by-pass around the proline pool, an experiment was performed in which C^{12}-proline was first added to establish a pool of unlabeled amino acid. After 1 min, a small quantity of C^{14}-proline of high specific radioactivity was added without appreciably altering the proline concentration or the steady pool size.

Figure 3 shows the results of such an experiment. As the amount of proline in the pool is constant, the specific radioactivity of the pool is simply proportional to the measured total radioactivity of the pool (P in Fig. 3). Since the rate of entry of proline into protein is also constant, the rate of entry of C^{14}-proline into the protein will be proportional to the radioactivity of the pool, if the pool is *the* source of proline for protein synthesis. The shape of the measured curve for incorporation of label into protein agrees remarkably well with the curve calculated on this assumption.

If even a few per cent of the proline entering the protein had by-passed the pool, as suggested by the upper drawing in Fig. 2, it would have

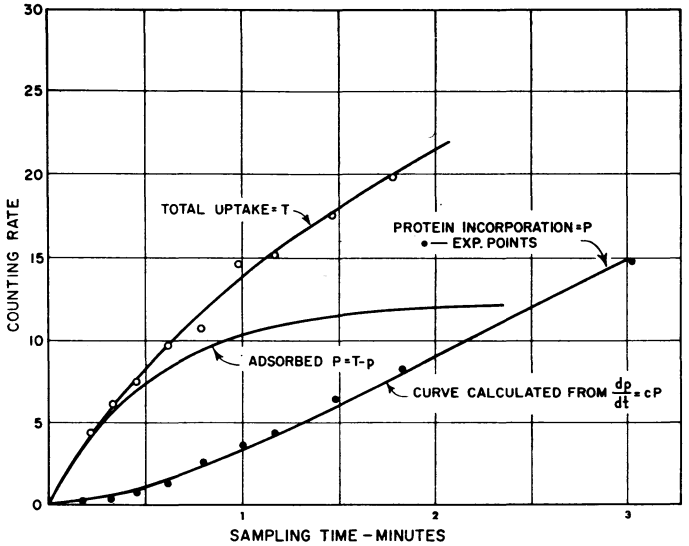

FIG. 3. *C^{12}-proline (0.8×10^{-6} M) was added 1 min before the carrier-free C^{14}-proline. An amount of medium was added with the C^{14}-proline such that there was no change in proline concentration.*

caused a detectable initial rise in the protein incorporation curve. This experiment demonstrates that passage through the pool is an obligate step in the incorporation of exogenous proline into the protein. The precision with which the experimental data fit the calculated curve also shows that pool amino acid is utilized for protein synthesis at random. The selection of an amino acid molecule is independent of the length of time the amino acid has been in the pool. If the amino acid previously existing in the pool had an advantage in this respect, there would be a further delay of incorporation of tracer into the protein.

A similar experiment has been performed with C^{14}-valine with identical results (Fig. 12). While no other amino acids have been tested to this degree of precision, the lack of conflicting evidence from many experiments with other amino acids indicates that this is a valid general conclusion for *E. coli*.

It cannot, of course, be concluded from such an experiment that internally synthesized amino acids do not by-pass the pool to some extent. That such a by-pass may operate is suggested by the failure of lysine and aspartic acid, even at very high external concentrations, to substitute completely for the internally synthesized amino acid in protein synthesis (14). A similar phenomenon occurs in the case of citric acid (14, p. 199). The definite proof of such an internal by-pass would be worth while, since it would imply that the pool of amino acid concentrated from the medium is organized within the cell in a way different from at least part of the pool of internally synthesized amino acid.

D. Failure to Observe Peptide Intermediates

When small quantities of amino acids of high specific radioactivity (*Chlorella* protein hydrolyzate containing 10% C^{14}) are supplied to the cells, very rapid uptake into the pool is observed. Chromatographic analysis of trichloroacetic acid or alcoholic extracts of samples taken at intervals indicates that certain amino acids (those with small native pools) are very rapidly incorporated into the protein and that others, such as glutamic acid, are completely incorporated into protein only after 10 to 15 min. The chromatograms do not show significant quantities of radioactive compounds other than the amino acids supplied.

In another type of experiment, cells that had exhausted their supply of glucose were given a small quantity of C^{14}-glucose (uniformly labeled) of very high specific radioactivity. Under these conditions, about 30% of the C^{14} incorporated is incorporated into macromolecules and 70% remains in the trichloroacetic acid-extractable pool. (A further discussion of this experiment appears below.) Chromatography of this pool shows that the principal part of the radioactivity occurs in the usual pool amino acids that occur in a growing cell. In addition, small quantities of glutamine and asparagine have been identified. Traces of several unidentified compounds are present, but these do not appear to be peptides.

The sensitivity of these experiments to intermediates present in trace quantities is high. For a number of the amino acids, the total quantity present in the pool (tracer plus native amino acid) corresponds to the amount utilized for protein synthesis in about 30 sec. Peptides containing these amino acids would be detected if the quantity corresponded to only a 1-sec requirement for protein synthesis.

It should be pointed out that these experiments do not eliminate the possibility of the occurrence of small peptides as intermediates in protein synthesis. Rather, they demonstrate that the trichloroacetic acid-soluble pool of these compounds is extremely small, if it exists at all. That such pools of peptides are very small or absent is also indicated by the rapidity of the incorporation of $S^{35}O_4$ into protein under conditions of sulfur starvation (12).

E. Requirement for Energy

Figure 4 shows the results of an experiment in which cells that had exhausted the supply of glucose several hours previously were supplied C^{14}-proline. The rate of pool formation is reduced by approximately a factor of 20. This residual rate is probably due to endogenous reserves of energy which slowly become available. When glucose is added along with the C^{14}-proline, pool formation begins instantly and protein synthesis is delayed for less than 1 min. Studies of pools formed in the presence of other amino acids indicate that the requirement for glucose (or some equivalent energy source) is quite general. At low concentrations, however, some of the other amino acids seem to be taken up to a greater extent than proline, in the absence of glucose.

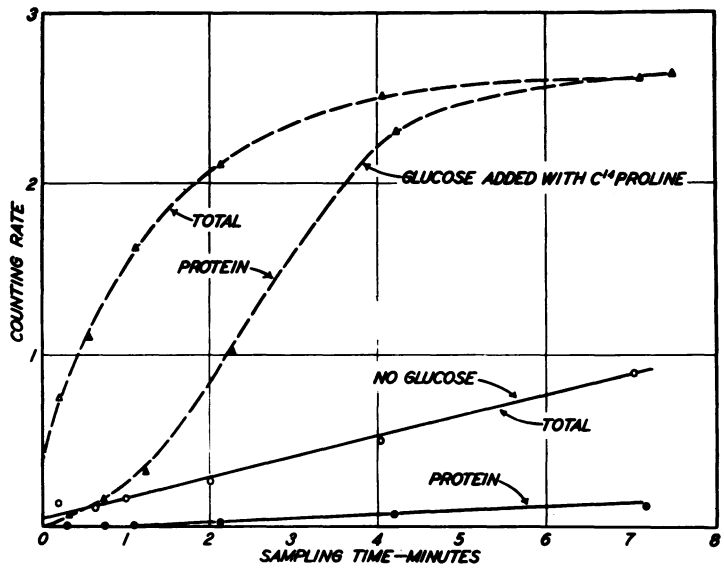

FIG. 4. *Effect of glucose on the incorporation of C^{14}-proline. Lower curves (solid line) show the incorporation in the absence of glucose. The upper curves (dashed line) show the incorporation when 0.1% glucose was added with the C^{14}-proline. C^{14}-proline concentration, 0.28 × 10^{-6} M; cell concentration, 0.07 mg, dry weight, per ml; temperature, 37 C.*

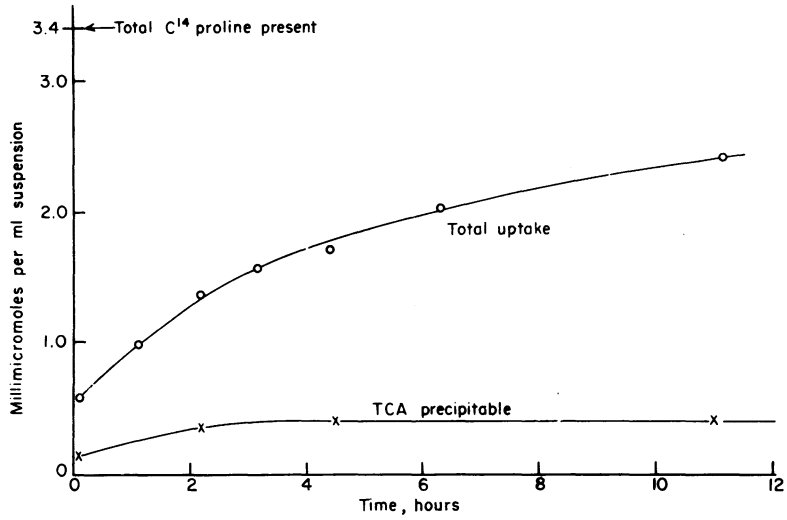

FIG. 5. *Proline pool formation at 0 C, 1.1 mg of wet cells per ml of suspension; 3.4 × 10^{-6} M C^{14}-proline. Exponentially growing cells were chilled to 0 C for 45 min before C^{14}-proline was added. TCA = trichloroacetic acid.*

Figure 5 shows the kinetics of proline pool formation at 0 C. Note that the abscissa scale is in hours, not minutes. As might be expected in an energy-requiring process, the rate of pool formation is very low. Both in the absence of glucose and at 0 C, pools are formed very slowly, but pre-existing pools are maintained for long periods of time. Further experiments on the loss and exchange of pools under these conditions are described below.

The concentration of the pool amino acid, calculated over the whole cell volume, may be several thousand times the external concentration. Some source of energy is necessary to establish such a concentration difference. It could, however, come immediately from reactions coupled to glycolysis or have been stored previously in sites for adsorption of the amino acid. The former alternative is supported by the requirement for glucose. The fact that endogenous reserves do not in general supply the small amount of energy required, of course, could not have been predicted.

While pools are formed very slowly in the absence of glucose or at 0 C, preformed pools, if they are not too large, are nevertheless maintained for long periods of time. An experiment described below (Fig. 15) shows the effect of the exhaustion of glucose after formation of a proline pool. In this case, the pool was maintained for 20 min without change. Other experiments show that such pools are maintained for many hours. At 0 C, pools that have been formed before the cells have been chilled are maintained for days.

It has been found that when a very large pool (near saturation) is formed at 25 C, a considerable fraction of the pool is lost at the time of chilling to 0 C. The remaining part of the pool, however, appears to be stable for long periods. The stability of very large pools in the absence of glucose at 25 C has not been tested.

In one type of experiment, growing cells were allowed to exhaust the glucose supply, and then C^{14}-glucose of very high specific radioactivity was added in an amount sufficient to support growth for only a few minutes. Seventy per cent of the incorporated radioactivity quickly appeared in the trichloroacetic acid-soluble fraction and remained there without significant change for several hours. After ½ hr, samples of the trichloroacetic acid-soluble pool and the medium were withdrawn and analyzed by chromatography. Large quantities of amino acids were found in the pool and traces in the medium. The ratios of the concentrations of the amino acids in the cells to the concentrations in the medium were evaluated; they ranged from 28,000 for valine, 14,000 for glutamate, and 7,300 for proline to 2,300 for aspartic acid. Again, it is clearly shown that the cell has the capacity in the absence of glucose to maintain a highly concentrated metabolic pool.

F. Lability of the Pool

A large part of the pool is lost when the cell is damaged in almost any fashion. Mild treatments which do not interfere with subsequent growth of the cell may cause the pool to be lost completely. On the other hand, the deprivation of most nutrients does not cause the pool to be lost. Pools may be formed under a variety of conditions that block synthesis (such as nitrogen starvation or the presence of chloramphenicol) so long as an energy source is present.

Table 1 presents the results of a study of the stability of the proline pool in the presence of various reagents at 0 C. Cells containing an unlabeled proline pool were chilled to 0 C, and the pool was labeled by exchange as described in the section on zero-degree exchange. Samples of these cells were then treated and assayed as described in the legend to the table. The data in this table show that the pool is released from the cells by mild treatment from a chemical point of view, such as small shifts in pH or moderate concentrations of ethanol. If chemical bonds are involved in holding the pool, they are extremely labile.

Physical damage to the cells also releases the pool. As shown in Table 1, freezing and thawing release part of the pool, although the cells grow with little lag after this treatment. A water wash will remove the pool entirely and, after this treatment, the cells will grow normally within 2 min after restoration to the normal medium. Details of the effects of osmotic shock are described below. Any of the methods that have been used to disrupt the cells also cause the pool to be released. Examination by chromatography shows no sign of chemical modification of amino acids that have been concentrated in the pool, except for conversions occurring in the normal pathways of amino acid synthesis. Only traces of pool amino acids have been found in association with macromolecules after disruption of the cells.

G. Specificity of Pool Formation

Small proline pools are entirely uninfluenced by other amino acids at relatively high concentrations. Figure 6 shows the uptake of C^{14}-proline under the same conditions as those for the experiment shown in Fig. 1, except that 15 other amino acids were added, each at 100 times the concentration of the proline. The pool size and initial rate of pool formation are identical with

TABLE 1. *Extraction of proline pool at 0 C*[a]

Added reagent or procedure	Final concentration or pH	Trichloroacetic acid-soluble proline extracted
		%
Trichloroacetic acid	5%	100
Trichloroacetic acid	0.25%	20
Ethanol	10%	0
Ethanol	20%	47
Ethanol	30%	95
Ethanol	40%	107
Butanol	10%	90
Toluene	Saturated	20
Pyridine	1%	0
Roccal	0.5%	100
Dinitrophenol	0.002 M	40
Glucose	10%	0
NaCl	10%	35
NaOH	pH 10.5	116
NaOH	pH 8.1	40
NaOH	pH 7.7	10
HCl	pH 6.5	0
HCl	pH 5.5	26
HCl	pH 4.7	50
HCl	pH 4.3	60
HCl	pH 2.8	47
HCl	pH 1.8	101
HCl	pH 1.0	100
Chill to −80 C and thaw	Once	25
	Twice	37
Sonic disintegration to reduce optical density at 650 mμ by 70%		80

[a] Samples of a suspension in exchange equilibrium were added to tubes at 0 C containing reagents in the proper amounts to bring the final suspension to the condition described in the second column. After 10 min these suspensions were filtered, and the fraction of the trichloroacetic acid-soluble proline that had been extracted was calculated from the radioactivity of the precipitate.

those shown in Fig. 1. The time required for completion of the incorporation into protein is extended by about 30%. This difference is probably due to the presence of proline impurity (to the extent of 0.02%) in the mixture of other amino acids.

This result clearly demonstrates a high degree of specificity. It must be pointed out, however, that the proline pool size and rate of formation increase with the external concentration at this low concentration (see below). As a result, carrier proline itself would have to be added at several times the concentration of the C^{14}-proline in order to reduce appreciably the amount of C^{14}-proline appearing in the pool at any time. This situation merely reduces the sensitivity of the test for certain types of interference by other amino acids. Since the other amino acids were present at 100 times the proline concentration, it may still be concluded that they have very little affinity for the specific mechanisms for proline pool formation.

The formation of very large proline pools is, however, interfered with by other amino acids. At high concentrations of proline, the proline pool saturates at a value of about 240 μmoles per g, dry weight. In the presence of high concentrations of other amino acids, the maximal proline pool is reduced by a large factor. A similar conclusion was reached from quite a different type of experiment. The maximal pool size for proline rises to 1,000 μmoles per g of dry cells in media of high osmotic strength (see discussion of osmotic properties of the pool, below). Under the same conditions, however, the total pool for all amino acids formed from casein hydrolyzate (20 mg per ml) was measured to be only 1,000 μmoles per g, dry weight.

It thus appears that there are specific mechanisms for the formation and maintenance of small amino acid pools and less specific, or completely nonspecific, mechanisms for the formation of very large pools. It would be possible to measure the maximal size of the specific and nonspecific pools by examining in detail the influence of other amino acids as a function of concentration, but this work has not been carried out.

Whereas there appears to be a concentrating system which is entirely specific for proline, there are other concentrating systems which apparently function for groups of similar amino acids (7, 8). Our observations on the interactions among one of these groups (valine, leucine, and isoleucine) will be discussed in the following paragraphs.

When carrier-free valine is supplied, the cells almost entirely remove it from the medium within about 10 sec, as shown by the upper curves in Fig. 7. The label is also very rapidly incorporated into protein. The effect of a moderate concentration of isoleucine (2.3×10^{-6} M) is

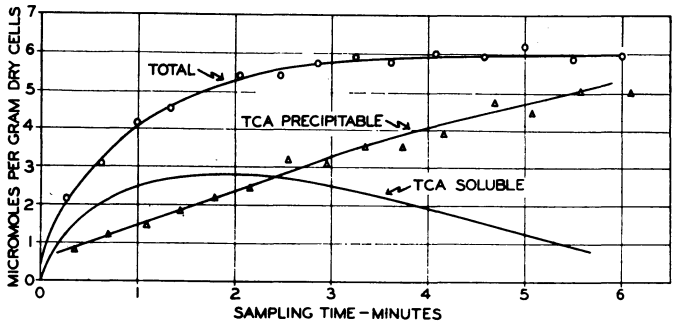

FIG. 6. *Incorporation of C^{14}-proline in the presence of other amino acids. The suspension was identical with that of Fig. 1 with the addition of 0.013 mg per ml (about 10^{-4} M) of each of the following: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine. TCA = trichloroacetic acid.*

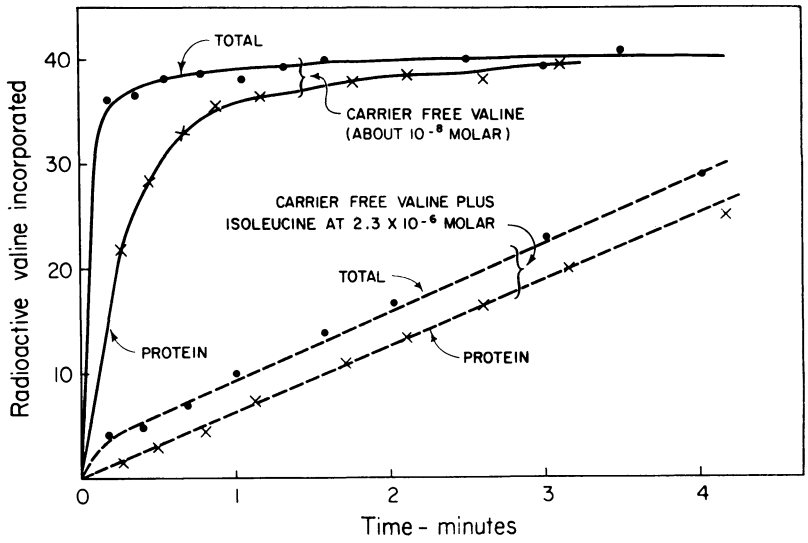

FIG. 7. *Effect of isoleucine on the incorporation of valine at very low concentration: 0.125 mg per ml dry weight, of cells growing at 25 C. Upper curves (——), carrier-free C^{14}-valine, about 10^{-8}, M. Lower curves (– – –), carrier-free valine plus C^{12}-isoleucine at 2.3×10^{-6} M.*

shown in the lower curves. The isoleucine reduces the rate of incorporation of the valine into the cell by more than a factor of 50. In this case, the quantity of valine supplied is so small that it does not significantly alter the size of the previously existing valine pool. One simply observes the rate of entry of the tracer valine into the pool through the pool-forming mechanism and its entry into protein after dilution by internally synthesized valine in the pool. For both examples in Fig. 7, the label present in the pool is utilized at a rate such that it would be exhausted in about 30 sec if more were not flowing in. Thus, the native valine pool equals 30 sec worth of valine requirement for protein, or 1.5 μmoles per g of dry cells.

The strong suppression of the rate of valine uptake by the isoleucine indicates that there is a common mechanism for the concentration of the two amino acids by the cell. In addition, an important feature of this concentrating mechanism is demonstrated by the strong interference at low concentrations where both amino acid pools are far below their saturation values. This result is most simply interpreted as an interference between the two amino acids at a cata-

TABLE 2. *Summary of the interactions of isoleucine, leucine, valine, and related compounds during pool formation*[a]

Labeled compound	Concentration	Competitor	Concentration	Suppression of pool	Suppression in rate of incorporation into protein
	μg/m		μg/ml	%	%
1. Isoleucine	0.29	Leucine	8.8	98	75
2. Isoleucine	0.17	Leucine	9.1	98	82
3. Isoleucine	0.29	Valine	8.8	91	75
4. Isoleucine	0.17	Valine	9.1	94	75
5. Leucine	0.02	Valine	3.6	>60	
6. Leucine	0.29	Valine	10.0	86	10
7. Leucine	0.29	Valine	10.0	90	35
8. Leucine	0.29	Isoleucine	10.0	95	45
9. Leucine	0.32	Norleucine[b]	9.6	0	0
10. Leucine	0.32	Norvaline[b]	9.6	67	58
11. Valine	0.30	Leucine	9.1	80	73
12. Valine	0.007	Isoleucine	0.3	90	92
13. Valine	0.02	Isoleucine	10.0	94	>95
14. Valine	0.15	Isoleucine	0.075	16	20
15. Valine	0.15	Isoleucine	0.15	32	39
16. Valine	0.15	Isoleucine	0.30	54	54
17. Valine	0.15	Isoleucine	1.5	85	89
18. Valine	0.10	Isoleucine	4.0	87	90
19. Valine	0.30	Isoleucine	10.0	>90	>95
20. Valine	0.30	D-Valine	10.0	0	0
21. Valine	0.30	Norleucine[b]	10.0	65	0
22. Valine	0.30	Norvaline[b]	10.0	84	84
23. Valine	0.29	α-Ketoisovalerate	0.29	+18[c]	30
24. Valine	0.29	α-Ketoisovalerate	0.87	+35[c]	45
25. Valine	0.29	α-Ketoisovalerate	2.9	+65[c]	58
26. Valine	0.30	α-Ketoisovalerate	7.5		65

[a] The values for the suppression of the pool and reduction of the rate of incorporation of radioactivity into the protein are calculated from individual experiments such as those shown in Fig. 8.

[b] Norvaline at 10 μg per ml suppresses growth rate by 42%; norleucine at 10 μg per ml does not suppress growth rate.

[c] Increase in pool size.

lytic step in the pool-forming mechanism. In order for the strong interference to occur, however, this catalytic site must be nearly saturated with isoleucine at a concentration far below that at which saturation of the pool itself occurs for isoleucine. A similar conclusion can be drawn from the small variation in the rate of valine pool formation shown in Table 4.

Table 2 shows the results of a large number of experiments designed to explore the interactions in pool formation of valine, leucine, isoleucine, and a few related compounds. The data in this table were obtained from measurements of the kinetics of pool formation similar to those illustrated in Fig. 8. It is obvious that a common mechanism plays a part in the concentration of these three amino acids by the cell. Norleucine and norvaline also have some affinity, but D-valine has no measurable affinity for this step in the concentration process. Apparently α-ketoisovalerate is also concentrated by the cells, but by a separate mechanism. The reduction of the rate of incorporation of C^{14}-valine into protein (shown in the last column of Table 2) indicates that α-ketoisovalerate is converted into valine in the pool and thus dilutes the tracer. The last column in Table 2 shows the effect of the competing compounds on the rate of incorporation of label into protein. A reduction in this rate, except in the case just mentioned, results from a dilution in the pool of the C^{14}-amino acid by internally synthesized C^{12}-amino acid.

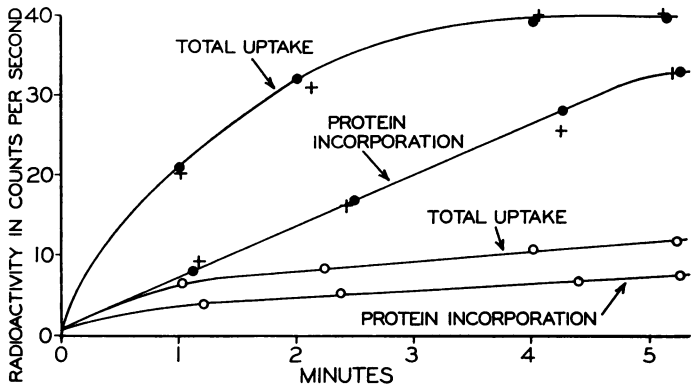

FIG. 8. *Typical experiment from which the data of Table 2 were obtained. For all the curves, 0.3 μg per ml of C¹⁴-valine was added to 0.08 mg, dry weight, per ml of growing cells at time zero. For the control (●), no competitor was present. For the curves marked (+), 10 μg per ml of D-valine were added at time zero. For the curves marked (○), 10 μg per ml of norvaline were added at time zero.*

Table 2 shows that a simple reciprocity does not occur in the interaction of isoleucine and valine. As shown in lines 19 and 3 of Table 2, the effect of isoleucine on valine is greater than that of valine on isoleucine. Line 15, however, shows that an equal quantity of isoleucine depresses the valine pool by only 30%.

Isoleucine acts, in effect, as a "dog in the manger" in suppressing the valine pool. Line 16 of Table 2 shows that when isoleucine is present at twice the valine concentration, the valine pool is suppressed to one-half its normal value at this concentration. In this concentration range, however, if the valine concentration is tripled, the pool is tripled. Indeed, the maximum pool size for either valine or leucine is at least 10 times the pool size at the concentration used. Another example leading to a similar conclusion has been discussed above.

In another type of experiment, in order to show the displacement of the pool by a competitor, leucine was added after a C¹⁴-isoleucine pool had been formed. Figure 9 shows the uptake of C¹⁴-isoleucine as control. Figure 10 shows the results of an initially identical experiment in which C¹²-isoleucine was added at 40 sec at 30 times the concentration of the tracer. With this concentration change, the pool does not increase in proportion to the concentration, and C¹⁴-isoleucine is removed from the pool by exchange. The specific radioactivity of the pool isoleucine immediately starts to decrease, and as a result, the rate of incorporation of C¹⁴-isoleucine into the protein decreases to a steady-state value one-thirtieth of the control.

Figure 11 shows the results of the corresponding experiment in which an equally large quantity of C¹²-*leucine* was added at 40 sec. The C¹⁴-isoleucine is removed from the pool by exchange with the leucine. During the 40 sec after addition, while a measurable quantity of isoleucine remains in the pool, the rate of isoleucine incorporation into the protein is unaffected. After this period, when the isoleucine pool has dropped to a very small value, the incorporation into protein continues at about one-sixth the rate of the control. Similar experiments have been performed for various combinations of valine, leucine, and isoleucine. In each case, the rate of incorporation into protein of the labeled compound from the pool is unaffected until the quantity in the pool drops to a low value. The degree of suppression of the pool and rate of protein incorporation are different in the various cases.

In concluding the discussion of specificity of pool formation, it is worth while to point out that a large number of concentrating systems specific for given compounds or groups of compounds are now known in *E. coli*. In addition to the amino acid systems, several have been identified for sugars and nucleic acid bases. The number that are known at present probably does not exceed a dozen, but if all possible low molecular weight metabolites were tested, the number of specific transport mechanisms would probably turn out to be many times larger. This does not

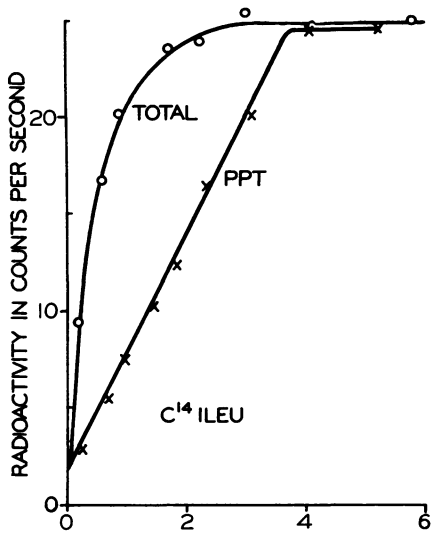

FIG. 9. *Leucine-isoleucine interaction, control; 0.3 μg per ml of C¹⁴-isoleucine was added at time zero to 0.25 mg, dry weight, per ml of growing cells.*

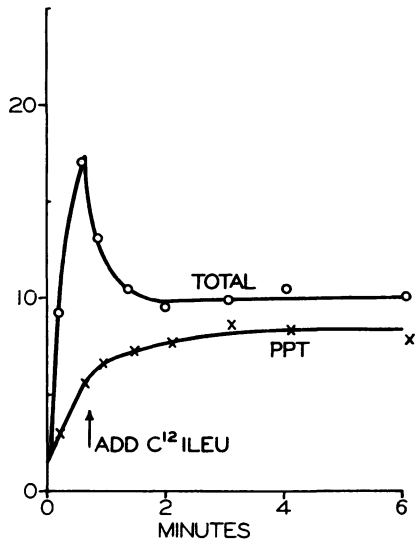

FIG. 10. *Leucine-isoleucine interaction; effect of carrier isoleucine. Same as Fig. 9 with 10 μg per ml of C¹²-isoleucine added at 40 sec.*

add very much to the known number of enzymes in bacteria, but when it is considered that each of these is coupled to an energy-supplying system, it nevertheless becomes an impressive array. The implication is that concentrating systems have been important in the evolution of bacteria. Further, if they are all located in the cell membrane, as has been suggested, and there are many sites for each function, the membrane is indeed a complex structure.

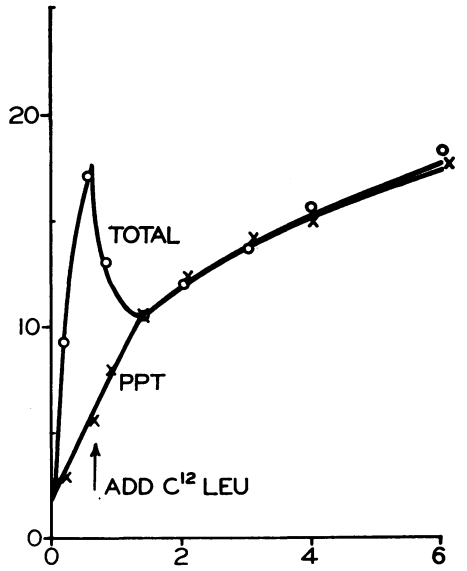

FIG. 11. *Leucine-isoleucine interaction; effect of leucine competitor. Same as Fig. 9 with 10 μg per ml of C¹²-leucine added at 40 sec.*

H. Exchange between the Pool and the Environment

In Fig. 12 are shown the results of an experiment performed to measure the rate of exchange when there is a steady flow of amino acid through the pool (see also Fig. 3). C^{12}-valine (2.5×10^{-6} M) was initially added to a growing culture of cells. Four minutes later, when the pool had reached a steady value, as shown by control experiments, C^{14}-valine was added without appreciably altering the external concentration of valine.

That this experiment demonstrates exchange between the pool valine and exogenous valine may be seen by referring to the two drawings in Fig. 13. The drawings represent the expected kinetics of labeling of the pool and protein when tracer is added to a system in which a constant-size pool has been previously established and where sufficient valine is present to maintain a constant pool for the duration of the experiment.

The lower drawing represents the case of no exchange. Molecules of labeled amino acid from the environment can only enter the pool as molecules leave the pool to enter protein. The total

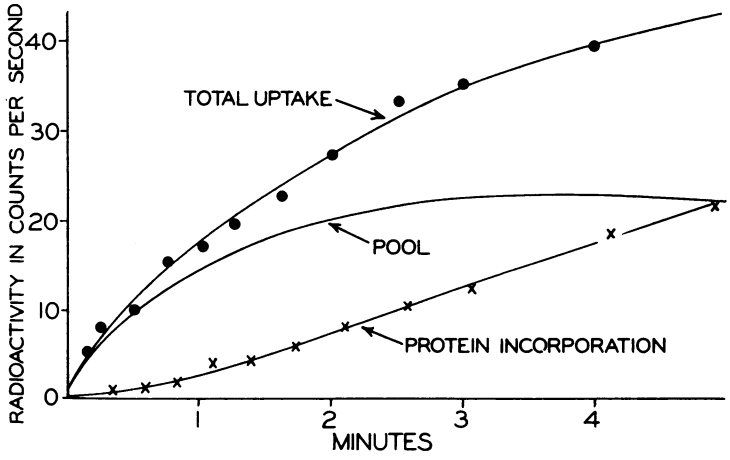

FIG. 12. *Demonstration of exchange when a steady pool has been established. Growing cells (0.07 mg, dry weight, per ml) were supplied 0.3 μg per ml of C^{12}-valine 4 min before the addition of the C^{14}-valine. At the time C^{14}-valine was added, the unlabeled pool had reached a steady state. The rapid initial incorporation of C^{14}-valine shows that exchange is occurring between pool valine and external valine.*

label in the cell therefore rises linearly from the time of addition of the tracer. The specific radioactivity of the pool slowly rises as C^{14}-valine enters the pool, and the rate of entry of the label into protein rises in proportion to this specific radioactivity.

The upper drawing represents the expected kinetics of labeling when exchange occurs at a rate considerably greater than the rate of utilization for protein. In this case, labeled amino acid enters the pool initially at a high rate. Later, when the specific activity of pool amino acid equals that present externally, labeled amino acid enters the cell at just the rate at which it is utilized for protein synthesis. It is clear that the upper pair of curves is very similar to the pair shown in Fig. 12, and therefore it may be concluded that exchange is an important process for the entry of labeled valine into the pool. Observations with other amino acids indicate that this is a general phenomenon during pool formation in *E. coli*.

A simple calculation shows that the radioactivity of the pool valine should vary with time according to the relation, $P^* = P_E^* (1 - e^{-t/T})$, where P_E^* is the value after the specific activity of the pool valine equals that in the medium. The time constant, T, depends on the rate of incorporation of valine into protein and the rate of exchange between pool and external valine. The experimental curve of Fig. 12 fits this relation very accurately, and T = 71 sec. The time constant expected if there were no exchange would have been 241 sec.

The rate of exchange may be expressed more simply as shown in Fig. 14. The rate of entry of labeled amino acid into the pool is more than 3 times the net flow of amino acid through the pool.

An exogenous supply of energy (glucose) is not necessary for exchange to occur. Figure 15 gives the results of an experiment in which nonradioactive proline and a limited supply of glucose were added simultaneously to two identical cultures. C^{14}-proline was added to culture A at zero time and to culture B at 13 min. In both cultures the glucose was completely exhausted at 10 min. Experiment A (solid line) shows that the pool does not increase after the glucose is exhausted, but is maintained at a constant size. Protein synthesis also ceases at the time when glucose is exhausted. Experiment B (dashed line) shows, however, that exogenous proline enters the constant-size pool at a rapid rate. (The lack of entry of C^{14}-proline into the protein in B demonstrates that the glucose was indeed exhausted.) It is clear that exchange occurs between the proline in the pool and exogenous proline in the absence of an energy source. This experiment also suggests that the rate of exchange is not influenced by the presence of glucose. The experi-

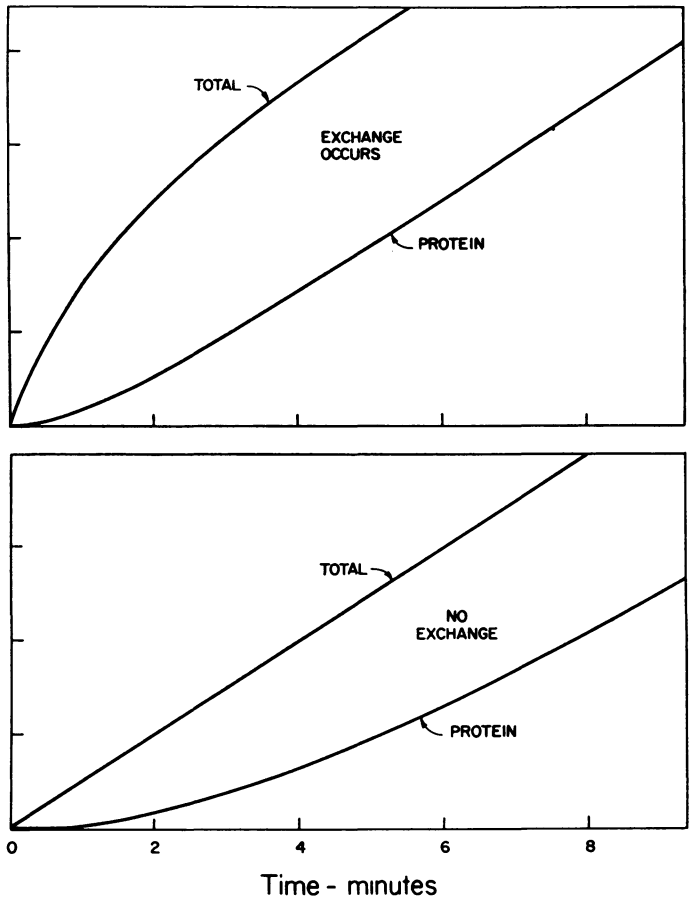

FIG. 13. *Kinetics of incorporation of labeled amino acid into cells with a pre-existing pool. These curve were calculated for a pool size and rate of protein synthesis comparable to those of Fig. 12. It was assumed that no change in pool size occurred at the time the tracer was added or at later times. The lower pair of curves is for the case of no exchange. The upper pair of curves was calculated for an exchange rate comparable to that implied by Fig. 12. The radioactivity of the pool is the difference between the total and protein curves, in each case.*

ments, however, have not been performed with sufficient accuracy to establish this point.

At 0 C, exchange occurs, while pool formation is very strongly suppressed. In order to study exchange at 0 C, a pool of the appropriate size must be formed before the suspension is chilled. Such pools have usually been formed with unlabeled amino acids so that radioactivity does not enter the protein. Figure 16 gives the results of an experiment in which growing cells were suspended in unlabeled proline for 2 min at 24 C and then chilled to 0 C, the chilling process taking about 5 min. C^{14}-proline was then added to the system, and a series of samples was taken. Figure 16 shows that the labeled proline entered the trichloroacetic acid-soluble fraction of the cell, but almost no incorporation into the trichloroacetic acid precipitate occurred. It appears that the external C^{14}-proline exchanged with the C^{12}-proline that was previously adsorbed at 24 C and had remained in the pool during the chilling process. To show that exchange was occurring, a small amount of C^{12}-proline was added after equilibrium had been approached. The amount of C^{14}-proline in the pool then fell as a new exchange equilibrium was approached.

Table 3 shows the relative suppression of proline pool formation and exchange at 0 C for a given external concentration of proline.

If C^{12}-amino acid is not added before the sus-

pension is chilled, exchange is observed with the "native" pool that normally exists in the cell in the absence of supplement. In the case of proline, this very small pool has been estimated by means of exchange measurements at 0 C to be about 0.5 μmole per g, dry weight. This result agrees with estimates made with growing cells at 25 C. In the case of valine, however, the native pool estimated by exchange at 0 C appears to be much larger than the native pool observed in growing cells at 25 C. This is in part due to the fact that valine can exchange with the leucine and isoleucine pools. In addition, however, excess valine appears to be synthesized by the cells during the cooling process. The excess valine was observed by chromatography of the pool of chilled cells. That pool formation did not occur at 0 C was indicated by a detailed study at that temperature of the quantity of C^{14}-valine appearing in the pool as a function of the concentration of external C^{14}-valine. The resulting data fitted exactly a curve calculated on the basis of exchange with a valine pool of 15 μmoles per g of dry cells. It is something of a mystery that the

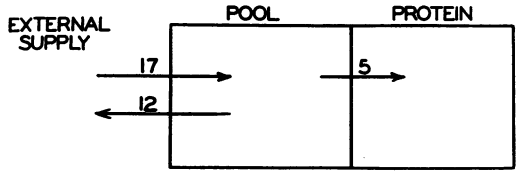

FIG. 14. *Schematic diagram showing the exchange of pool valine during incorporation at a concentration of 0.3 μg per ml. The numbers are flow rates in micromoles per gram of dry cells per 100 sec, calculated from the curve shown in Fig. 12.*

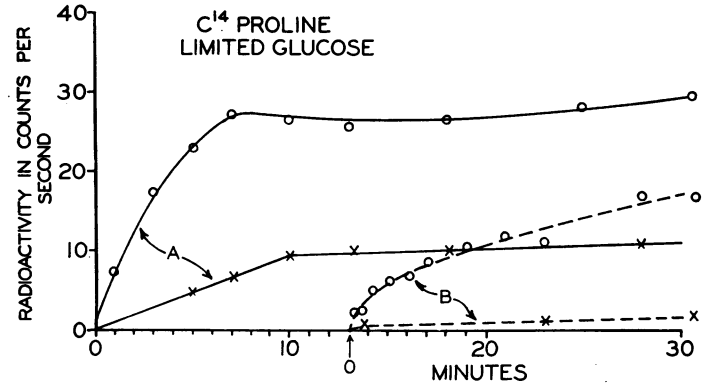

FIG. 15. *Maintenance and exchange of pool proline in the absence of glucose. In both experiments, growing cells were suspended at time zero in medium containing 10 μg per ml of glucose and 0.87 μg per ml of C^{12}-proline. For curve A, a small quantity of C^{14}-proline was added at time zero. For curve B, an equal quantity of C^{14}-proline was added at 13 min. In each case the upper curve (○) represents the total C^{14}-proline taken up, and the lower curve (×), the C^{14} incorporated into protein. The difference is the C^{14}-proline in the pool.*

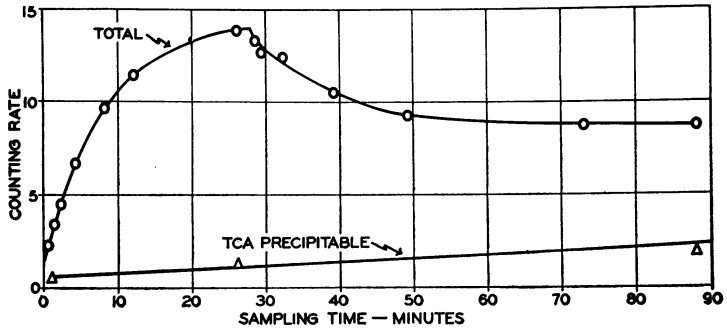

FIG. 16. *Exchange between pool and exogenous proline at 0 C. At 28 min, C^{12}-proline was added. Circles represent total incorporation; triangles, incorporation into trichloroacetic acid (TCA) precipitate.*

TABLE 3. *Approximate rates of formation and exchange*[a]

Temperature	Rate of	
	Pool formation	Exchange
C		
0	0.0074	0.18
25	2.0	0.63

[a] Results are expressed as millimicromoles of proline formed or exchanged per min per mg of wet cells at an external C^{14}-proline concentration of 3.5×10^{-6} M. No glucose was present in the exchange experiments.

cells, during the cooling process, synthesize an excess of valine equal to several minutes of synthesis at the normal 25 C rate. Perhaps this point should be explored further. There is, however, no question that the suppression of amino acid pool formation at 0 C is a general phenomenon in *E. coli*.

In this context, it must be mentioned that the experiments of Cohen and Rickenberg (7) on the concentration of amino acids by *E. coli* were performed by chilling the suspension to halt the concentration process. These experiments have given a good picture of the concentration process and the interactions of a number of amino acids, but some details may have been blurred by exchange in the cold during centrifugation.

As mentioned above, we have observed that very large pools are unstable at 0 C. The saturation value of the valine pool quoted in (7) and (8) is about 20 μmoles per g of dry cells, measured by chilling and then centrifuging the cells. Using the filter technique, we have observed a saturation value of 60 μmoles per g of dry cells (10^{-4} M external valine concentration). This factor of 3 may, in part, be due to the different strains of cells used and to different temperatures. The osmotic strengths of the media were nearly identical, so that they should not have influenced the saturation pool size. It seems likely that a large part of the difference is, in fact, due to the different methods of measurement. It appears that the pool for a given amino acid is made up of more than one component (see Discussion below). The components have different specificity and exchange rates and presumably different stability toward chilling. As a result, the filter and the chilling techniques may very well emphasize quite different aspects of pool behavior.

The specificity of the exchange process at 0 C also has been examined. Radioactive proline is not displaced from a pool in exchange equilibrium at this temperature by the simultaneous addition of 15 other nonradioactive amino acids, each at 100 times the proline concentration. Thus, the exchange process at 0 C is just as specific for proline as the pool formation process at higher temperatures. Exchange at higher temperatures must also be specific, since it plays an important part in the kinetics of pool formation. Excess leucine and isoleucine displace radioactive valine. from a pool at 0 C in exchange equilibrium. The rate of displacement appears to be similar to that caused by the addition of excess C^{12}-valine. Here again, the specificity of the exchange process appears to be similar to that of the pool-forming process.

Studies of the rate of exchange as a function of external concentration and pool size give surprising results, which yield insight into the exchange process and supply strong restrictions on models of the pool mechanism. For several reasons the rate measurements have been carried out at 0 C. It is convenient experimentally, since there is little incorporation into protein and the pools are stable for many hours. In addition it is possible to vary the pool size and external concentration independently. During pool formation at higher temperatures, the pool size will rise rapidly to the value dictated by the external concentration. However, at 0 C, the pool size will not show significant change for several hours, even when it is far from its normal value for the external concentration.

For these experiments, pools of a desired size were formed with unlabeled proline at 25 C. The suspension was then chilled to 0 C. The cells were centrifuged and resuspended in unsupplemented medium, and after approximately 1 hr at 0 C, C^{14}-proline was added. Since the external quantity was small compared with the amount in the pool, a very efficient labeling of the pool by exchange was achieved. After a steady state (equal internal and external specific activities) was reached, the external concentration was brought up to a chosen value by adding C^{12}-proline. The time course of exchange was then followed by measuring the loss of trichloroacetic acid-soluble radioactivity from the cells. The variation with

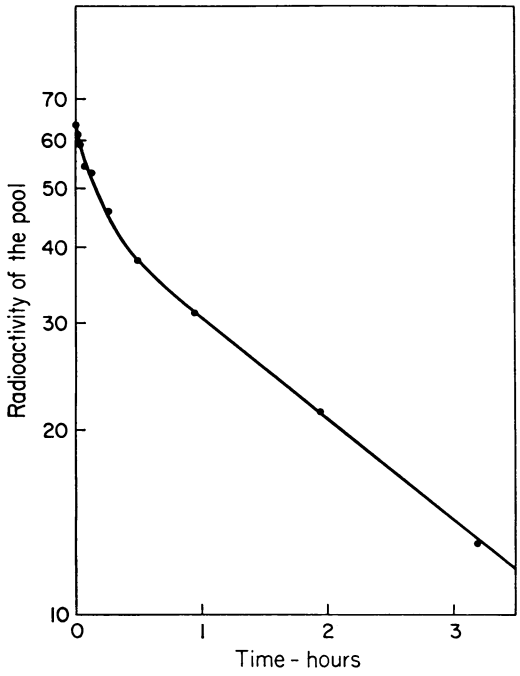

FIG. 17. *Time course of exchange of the proline pool at 0 C. The log of the radioactivity of the pool is shown as a function of time after C^{12}-proline was added (10^{-4} M) to a suspension containing a C^{14}-proline pool of 2.9 × 10^{-6} mole per gram, wet weight, in equilibrium with external C^{14}-proline (1.9×10^{-6} M) at 0 C.*

time of the logarithm of the radioactivity of the pool is shown in Fig. 17.

It is apparent that the time course of the exchange process does not follow a single exponential. It is possible, with good accuracy, to resolve this curve into two simple exponentials. When the results of such experiments over a wide range of concentration and pool size are examined, two components with widely different time constants can be resolved. A very definite conclusion can be drawn from these observations, owing to the simplicity of exchange processes. If a single, constant, homogeneous component exchanges with a constant quantity of amino acid in solution, the time course of the process must follow a simple exponential, i.e., display a single time constant This statement holds regardless of the nature and multiplicity of the mechanisms mediating the exchange, as long as the quantity of amino acid associated with the intermediate steps is small. For example, it might be suggested that there were separate fast and slow mechanisms which could act as intermediates in exchange between a single pool and the environment. However, the resulting time course would be a simple exponential with a time constant slightly faster than with the fast mechanism alone.

Thus it may be concluded that there are at least two separate components in the pool which are associated with the cell in different ways. The accuracy of these experiments is not sufficient to establish whether there are more than two components. The separate parts of the pool cannot exchange with each other at a rate faster than that shown by the slow component. The instability of the very large pools at 0 C suggests still another component in the pool.

The variation with total pool size of the exchange rates of both the fast and slow components is shown in Fig. 18. The number beside each point is the external concentration during exchange in micromoles per liter. It appears that the exchange rate is independent of the external concentration, except possibly at low concentrations.

The rapidly exchanging component of the pool is always smaller than the slowly exchanging one. It appears to saturate at less than 1.0 μmole per g of wet cells and is not easily observable when the total pool is greater than 10 μmoles per g. The exchange rate of the large, slow component is roughly proportional to the total pool size. Unfortunately, the accuracy of the data is not quite sufficient to determine whether the exchange rate of each of the components is proportional to its own size, although this result is suggested by the evidence.

The exchange process may occur either through reactions that are an essential part of the overall mechanism of pool formation or through reactions that play no real part in that process. In connection with the latter case, it should be noted that a reaction of the type,

$$A^* + R{=}A \rightleftharpoons A^*{-}R{-}A \rightleftharpoons A^*{=}R + A$$

where $R{=}A$ is some complex containing A, would be observable in an exchange study but would not necessarily be observable in the process of pool formation, since the reaction causes no net change in the amount of the complex.

Any satisfactory model of the amino acid pool must certainly allow for the occurrence of exchange in the absence of an energy source and

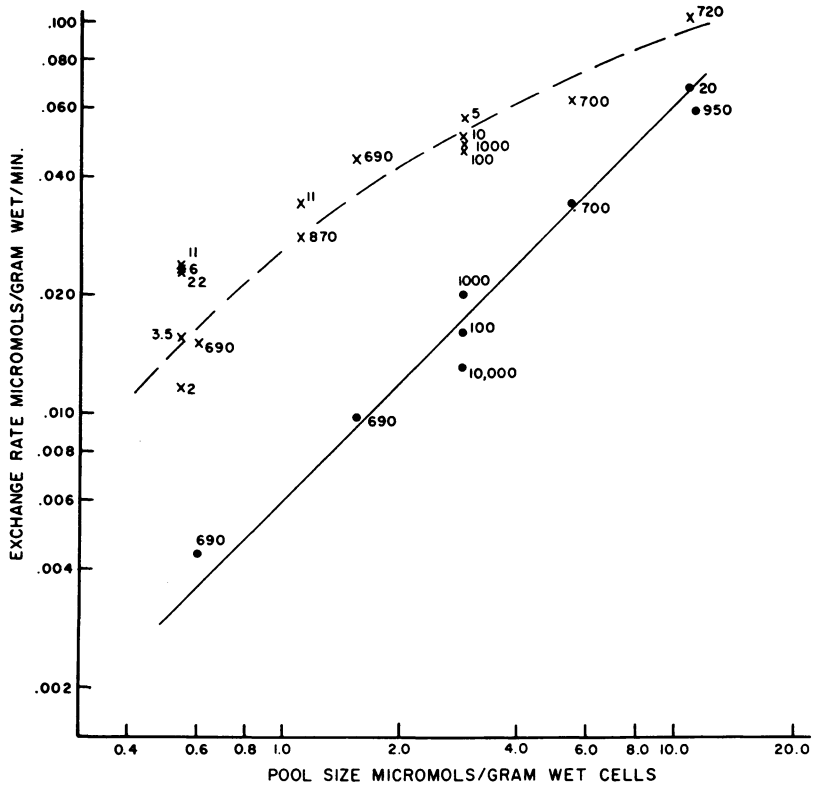

FIG. 18. *Rate of exchange as a function of pool size (log-log plot). The points were obtained from experiments such as that shown in Fig. 17 by fitting the time course of exchange to curves derived from the sum of two exponential decays. The numbers beside each point are the external concentrations during exchange, in micromoles per liter. The straight line shown would result if the exchange rate were proportional to pool size.*

for the strikingly different temperature dependence of the exchange process and the process of pool formation. A model of the pool mechanism should also have features which limit the rate of exchange and should suggest how the rate of exchange can be independent of the external concentration but proportional to the pool size. Finally, a sophisticated model should indicate how the different components of the pool differ in their association with the cell.

I. Variation of the Pool Size and Formation Rate with the External Concentration

Measurements of the pool size and the rate of formation of the pool as a function of external concentration are valuable since they may be compared quantitatively with calculations based on models. It would be in keeping with the traditions of enzyme chemistry if these obvious features of the pool could be pigeonholed by simply determining the Michaelis constants for the interaction of amino acids with the cell. However, pool formation is an energy-coupled process of a whole living cell, and it is not surprising that such simplicity is lacking.

A survey of a large number of exploratory experiments done for many other purposes indicated that the pool size did not rise quite in proportion to the external concentration at low concentrations (far below saturation of the pool). It also appeared that the rate of formation of the pool reached a maximum at an external concentration much lower than that at which the pool size reached its maximum value. There was, however, a large amount of scatter in the measurements of both the pool size and rate of formation. In order to avoid sources of variation, measurements of the kinetics of proline pool formation were carried out simultaneously at seven

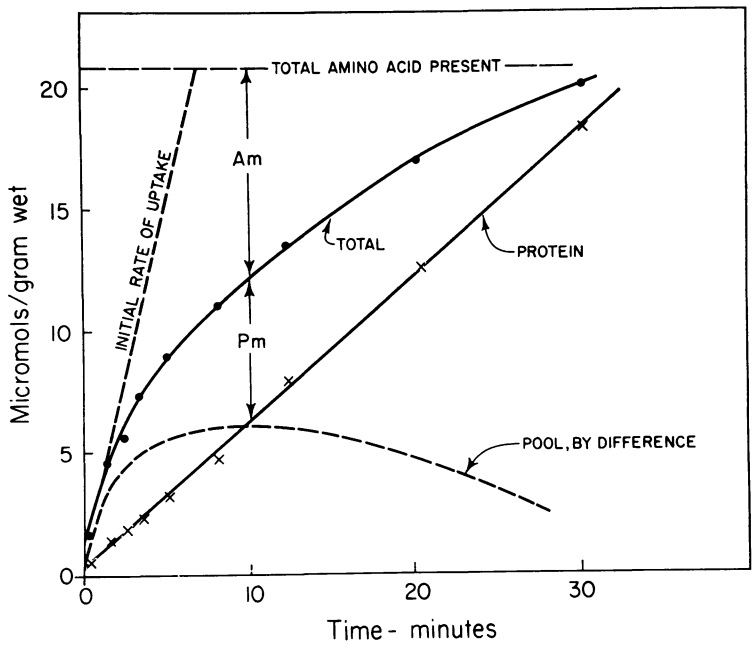

FIG. 19. *Kinetics of proline pool formation at 25 C. Exponentially growing cells (0.48 mg, wet weight, per ml) were supplied C^{14}-proline at 1.0 × 10^{-5} M. The initial total rate of uptake may be calculated from the dashed line. The maximum pool size and the external concentration at the time it was achieved are determined from the values, P_m and A_m, shown.*

different concentrations with samples from the same suspension of growing cells.

Figure 19 shows the kinetics of pool formation at an intermediate concentration (10^{-5} M). The lower curve shows the variation of the pool with time, achieving a maximum at about 10 min and slowly falling at later times. When the pool reaches its maximum value[1] (shown as P_m) its rate of change is zero, and therefore it has the steady value corresponding to the external concentration at that moment (shown as A_m).

The variation of the pool size with the external concentration determined from the seven simultaneous measurements is shown in Fig. 20. The dashed curve represents a classical adsorption isotherm (saturation value, S = 70 μmoles per g of wet cells; K_s = 4 × 10^{-5} M) fitted to the points at higher concentrations. At lower concentrations, the measured values of the pool are 3 times larger than those given by this isotherm. It is possible to fit the experimental curve with the sum of two isotherms. The results of a more thorough analysis are presented in the mathematical appendix. Thus the variation of pool size with concentration is consistent with the presence of more than one component in the pool. It does not, by itself, demonstrate this, since there is no independent evidence that individual components would follow the classical adsorption isotherm.

Two methods have been used to measure the initial rate of uptake of amino acid by the cells in this set of experiments. With the first method, a direct estimate of the total rate of incorporation was made from the early time points as shown by the dashed line in Fig. 19. By the second method, the difference between the maximum value of the pool and the pool at any time was plotted on logarithmic paper. A straight line results. In other words, the pool rises with time approximately as $P = P_m (1 - e^{-t/T})$. From the empirical time constant, T, and P_m, the initial rate of increase of the pool can be calculated. The initial total rate of uptake is determined by adding this figure to the rate of incorporation into protein. The two methods are in close agreement.

[1] See the mathematical appendix (Part IV) for a more precise method of evaluating the pool size at low concentrations.

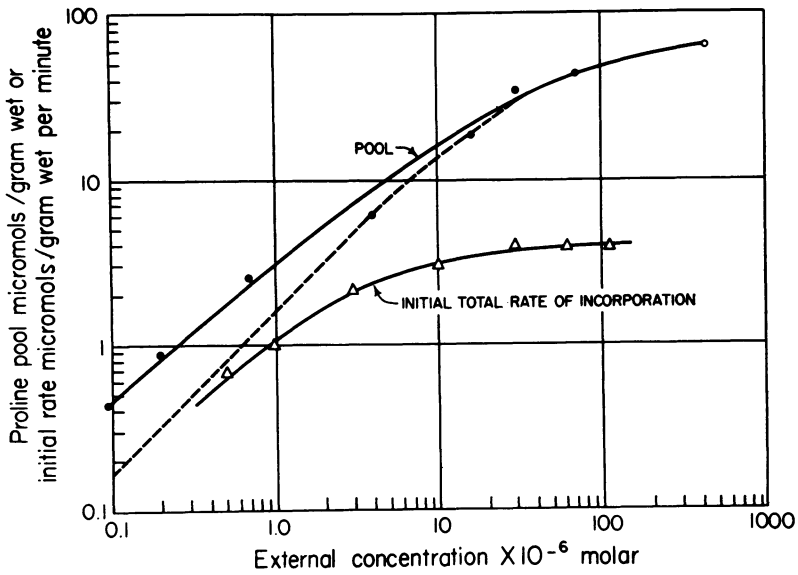

FIG. 20. *Log-log plot of the proline pool and initial rate of incorporation as a function of proline concentration at 25 C. The upper points (●) represent the results of a set of simultaneous measurements of pool size in one experiment as described in the text. The open circle (○) represents the results of a number of measurements of the saturation value of the proline pool. The dashed curve is an adsorption isotherm:* $P = \pi A/(A + k_s)$, *with* π = 70 *μmoles per g of wet cells and* $k_s = 4 \times 10^{-5}$ M. *The lower set of points (△) is the result of the measurement of the total initial rate of incorporation of proline.*

As the curves in Fig. 20 show, the variation of the initial rate of incorporation with concentration is much less than the variation of the pool size. Between 10^{-6} and 10^{-4} M, the pool increases by a factor of 15, while the initial rate of incorporation increases by only a factor of 4. The measurements of the initial rate of incorporation fit an adsorption isotherm (shown by solid line) with $K_s = 2.5 \times 10^{-6}$ M. The measurements of the pool size, at the larger concentrations, fit an adsorption isotherm (shown by the dashed line) with $K_s = 4 \times 10^{-5}$ M. Thus the pool itself saturates at a concentration more than 10 times the concentration at which the rate of pool formation saturates.

This pair of observations, by itself, is sufficient to eliminate the simpler models of the concentrating mechanism. As a result, it is worthwhile to consider whether any systematic errors are present which might weaken such an argument. With regard to the measurements of rate of incorporation, the process is relatively slow at the higher concentrations and there appears to be little source of error. However, at the lower concentrations, the rate falls rapidly during the first minute, and the measurements probably indicate rates that are somewhat slower than the actual rate at zero time. Again, for the pool size, the measurements at high concentrations are better, since the external concentration is changing slowly and one can be more certain of the external concentration at the time the pool has reached its maximum value. Since the very large pools are less stable, there is a chance that some part may be lost during the filtering process, but there is no evidence that this is so. At the lowest concentration used, the pre-existing native pool (measured to be 0.2 μmole per g of wet cells in this experiment) is significant compared with the labeled pool formed. In addition, internal synthesis continues (in the experiments at the lower concentrations) during the time required to form the pool. Thus the labeled amino acid is somewhat diluted in the pool. The pool size at low concentrations is therefore somewhat larger than that estimated by the method used for Fig. 20. When correction is made for these effects (see Fig. 31), the deviation of the measured curve from a single adsorption isotherm is increased. Further, since exchange occurs between the pre-existing pool and added amino acid, the measured total rate of incorporation is somewhat greater than that due simply to uptake of the amino acid. The lowest

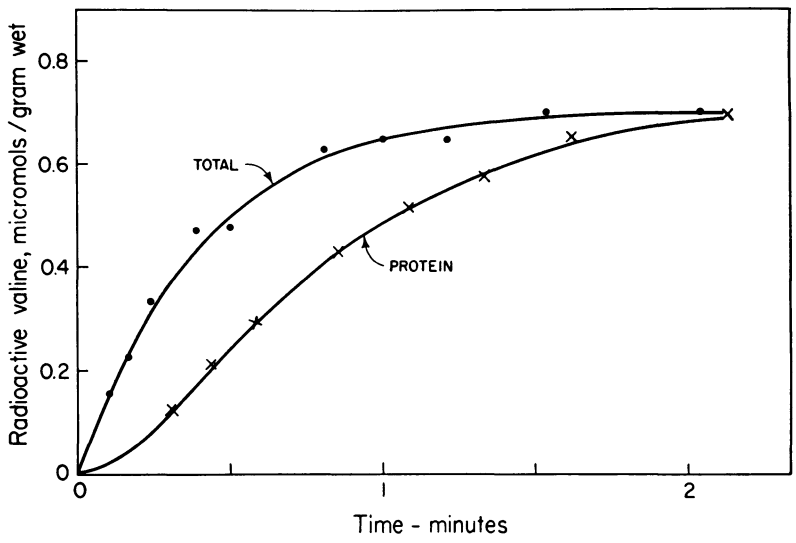

FIG. 21. *Kinetics of valine incorporation at a very low concentration. Exponentially growing cells (0.08 mg, wet weight, per ml) at 25° C were supplied C^{14}-valine at 5.6 × 10^{-8} M. The initial total rate of uptake is one-tenth of the maximum rate allowed by diffusion. The internal concentration, averaged over the whole cell volume, rises to 18,000 times that present externally.*

point (△) could not be in error by more than 50%, and this would not have a large effect on the apparent K_s.

It appears that the large difference between the concentrations at which the pool size and rate of formation saturate is definitely established and must be taken into account in building models of the process of pool formation.

To complete the comparison of the rate of formation and pool size, and for reasons of general interest, an example of the uptake of valine at an extremely low concentration is shown in Fig. 21. Valine at 5.6 × 10^{-8} M was supplied to an exponentially growing culture at 0.08 mg of wet cells per ml. The low cell density was used to reduce the rate of incorporation and the quantity of pre-existing valine in the suspension. The maximum rate of incorporation of C^{14}-valine into protein shows that at 30 sec, when the pool was maximal, the initial specific radioactivity had been diluted by about 30%. Thus there can be no major error in the original external concentration caused by valine pre-existing in the medium. The general shape of the curve is precisely that to be expected if a small unlabeled pool were initially present and valine continued to be synthesized by the cells. There is an initial lag in protein incorporation as the specific radioactivity of the pool rises, and then the rate at which C^{14}-valine enters the protein falls as the labeled pool is diluted by internal synthesis. Valine was chosen for this experiment, even though some confusion results from its conversion to leucine, because it was known to be incorporated extremely rapidly at low concentrations.

The initial rate of incorporation in this experiment, determined as above, was 2 μmoles per g of wet cells per min. This impressively high rate of uptake represents the removal of all of the valine in 600 cell volumes of medium in 1 sec by each cell. Calculation shows that the equilibrium rate of diffusion into a sphere of volume equal to one cell (continuously maintained at zero internal concentration) would result in the uptake of all of the valine in 5,400 cell volumes of medium per sec. Thus the flow of valine into the cell occurs at a rate about one-tenth of the maximum rate allowed by diffusion.

A few measurements of the incorporation of valine have been made at higher concentrations. Table 4 gives the results of measurements at the extremes of the concentration range that has been examined and of a pair in the median region. Values in parentheses are less certain. The pool size changes by a factor of 14, while the initial rate of incorporation changes by

TABLE 4. *Valine pool and rate of formation*

Valine concentration	Pool size	Initial rate of incorporation	Concentration ratio[a]
μmoles/liter	*μmoles/g wet*	*μmoles/g × min*	
70.0	15[b]		210
10.0	(10)	5.0	
2.9	6.5		2,200
1.3		3.0	
0.056	(0.7)	2.0	
0.016	0.3		18,000

[a] Obtained by: (pool per ml of cells)/(valine per ml of medium).

[b] Maximum pool.

only a factor of 2.5 over the concentration range from 0.056 to 10 μmoles per liter. In addition, the pool size measured at 0.016 μmole per liter is 5 times the value predicted by an adsorption isotherm fitted to the points at higher concentrations.

It is clear that for valine, just as has been shown previously for proline, the pool size as a function of external concentration does not follow a classical adsorption isotherm, and the concentration dependence of the initial rate of incorporation is very different from that of the pool size.

J. Loss from the Pool after Dilution of the External Amino Acid

A few exploratory measurements have been made of the rate of loss from the pool when the external amino acid concentration is suddenly reduced. These experiments show that the rate of loss from a given pool at 25 C is very much slower than its rate of formation. The existence of a rate of loss much slower than the rate of formation sets a stringent requirement for models of the process.

The rate of loss to the external environment is so slow that it cannot be measured when the pool is being utilized for protein synthesis. In the three experiments described below, protein synthesis has been inhibited in three ways: by reducing the temperature to 0 C, by removing glucose at 25 C, and by removing required supplements from a deficient mutant.

The experiment at 0 C is not easily interpretable, since all of the processes of pool formation and maintenance are strongly modified by the low temperature. Nevertheless, this experiment is worth discussing briefly. Figure 22 shows the results, and the legend describes the method used. A loss rate of 2% per min is observed in *C* and *D*. This rate is faster than that observed at 25 C (see below) from a somewhat larger pool. Curiously, it is also faster than the initial pool formation rate at 0 C (0.3% per min) measured in the experiment of Fig. 5. Since the pool sizes in these two cases are the same within a factor of about 2, this result is very difficult to explain. Until further experiments are carried out, only the qualitative result that the loss rate may be very different from the formation rate at 0 C can be used in arguments concerning the mechanism of pool formation.

In order to measure the rate of loss in the absence of glucose at 25 C, growing cells were supplied C^{12}-proline at 10^{-4} M for 10 min, centrifuged, and resuspended in the absence of both glucose and proline. Five minutes later, C^{14}-proline was added, and the pool rapidly became labeled by exchange (half-time to equilibrium, about 2 min). No incorporation into protein could be measured. Ten minutes later, when exchange equilibrium was established, samples were collected on collodion filters and washed continuously on the filter with unsupplemented medium for periods up to 10 min. There was a very fast initial loss of about 10% of the pool and no further measurable loss.

The measurement[2] at 25 C in the presence of glucose was carried out with the mutant 15 $T^-A^-U^-$ in the absence of its three required supplements (thymine, arginine, and uracil). Exponentially growing cells at 25 C (supplemented with arginine, thymine, and uracil) were harvested on a collodion membrane filter and washed with unsupplemented medium. The cells were immediately resuspended (0.5 mg of wet cells per ml) in the presence of glucose but without arginine, thymine, or uracil. It was felt desirable to complete the experiment during the 50 min (at 25 C) before thymineless death begins in this strain. Therefore, the C^{14}-proline was added about 10 min after the cells were resuspended in the absence of thymine. The

[2] While this section was being written, it became apparent that the point was important enough to require an additional experiment. The authors would like to express their appreciation to O. Maaløe at the Microbiological Institute of the University of Copenhagen for the use of the facilities at his laboratory and for the purchase of C^{14}-proline.

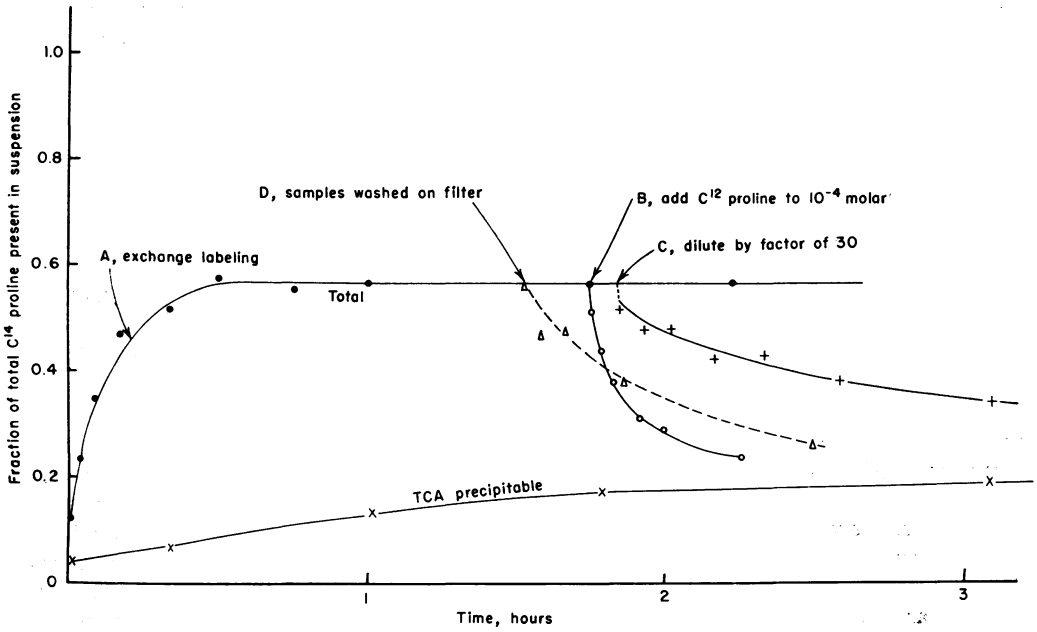

FIG. 22. *Study of rate of exchange and loss of C^{14}-proline pool in E. coli at 0 C. Cell concentration, 1.0 mg of wet cells per ml. The suspension was incubated at 25 C for 5 min after addition of 10^{-5} M C^{12}-proline and quickly chilled to 0 C. The cells then were centrifuged and resuspended in unsupplemented medium at 0 C. After 1 hr, C^{14}-proline was added in order to label the preformed pool by exchange (A). To an aliquot of the suspension (B) carrier proline was added to a concentration of 10^{-4} M. Another aliquot (C) was diluted by a factor of 30 with unsupplemented medium. Finally (D), samples were filtered and washed continuously on the filter for the times indicated, with unsupplemented medium at 0 C.*

upper curve (●) in Fig. 23 shows the kinetics of pool formation.

Eleven minutes after the C^{14}-proline was added, 20 ml of the culture were injected with a hypodermic syringe into 280 ml of medium supplemented only with glucose. The lower curve (+) shows that the total radioactivity in the cells hardly changes during the succeeding half hour. There is no measurable loss from the pool. The maximum possible loss rate consistent with the scatter in the measurements would be about 0.02 μmole per g of wet cells per min or 1% of the initial rate of formation.

These two experiments demonstrate that the rate of loss from the pool is very slow at 25 C when the external amino acid concentration is greatly reduced, whether or not glucose is present. Clearly, then, the outflow of amino acid from the pool must also have been small before the amino acid concentration was reduced.

This result implies that when a steady-state pool has been achieved, the circulating flow of amino acid between the pool and the medium must be small. In other words, the rate of entry of amino acid into the pool (which was initially large) must be strongly suppressed as the steady-state pool size is approached. In contrast, in a bimolecular chemical reaction, $A + B \rightleftharpoons AB$, the rate of formation of AB is constant as long as the concentrations of A and B remain constant. As equilibrium is approached, the rate of dissociation of AB rises until it equals the rate of formation.

In pool formation we are therefore dealing with an unusual type of process. A close analogy would perhaps be a centrifugal pump filling a reservoir (without significant leak) up to the limiting pressure that the pump can deliver. Such a pump might very well give a slow leak rate if the supply pressure were suddenly reduced. Most simple models for the concentrating process do not have this feature. The fairly complex carrier model, described later, does behave in just this way.

We should be reminded that exchange experiments, on the contrary, indicate a large circula-

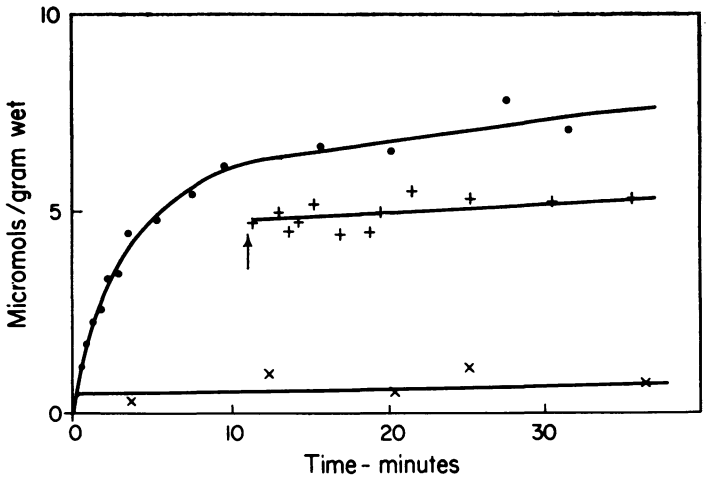

FIG. 23. *Stability of the proline pool after reduction of the external concentration. E. coli strain 15 T⁻A⁻U⁻ suspended at 0.5 mg, wet weight, per ml at 25 C in the absence of thymine, arginine, and uracil. Glucose was present at 2 mg per ml. At time zero C¹⁴-proline was added (●) to a concentration of 10^{-5} M (20 μmoles per g of wet cells). At 11 min (shown by arrow), part of the culture (+) was diluted by a factor of 15 and correspondingly large samples were taken. The concentration of C¹⁴-proline after dilution was 5 × 10^{-7} M. The incorporation into protein is shown by the lower set of points (×). The initial rate of uptake was 1.5 μmoles per g of wet cells per min. The rate of loss from the pool is not measurable, but certainly less than 1% of the rate of uptake.*

tion of amino acid when a steady pool exists at 25 C. Clearly this *must* occur by a mechanism independent of the *active* concentration process. The independence of the exchange process has been directly demonstrated at 0 C (Table 3), where the exchange rate is 25 times the formation rate. A large excess of exchange rate over formation rate is also observed at 25 C in the absence of glucose.

K. Osmotic Properties of the Pool

During the course of studies of the extraction of the amino acid pool by various agents, it was observed that distilled water completely removes the amino acid pool (5). However, when the bacteria were returned to their normal growth medium after treatment with distilled water, they almost immediately (within 2 min) resumed their normal rate of protein synthesis and rate of uptake of amino acids into the pool. These observations led to an experimental study of the osmotic properties of the pool.

Pool removal as function of osmotic strength of washing medium. A rapidly growing culture of *E. coli* cells was supplied C¹⁴-proline and, after the pool size had reached an approximately steady value, a series of samples was withdrawn and filtered on collodion membrane filters. The thin layer of cells collected on the filter (1 to 2 mg of wet cells) was then washed by drawing 2 ml of a given solution through the filter in 5 to 10 sec. Assays of the radioactivity of unwashed cells and of those washed with unsupplemented growth medium showed no significant difference, indicating that the quantities of amino acid in the pool were very large compared with the holdup of labeled medium on the filter and cell pad, and that little loss from the pool occurred. The fraction of the pool removed was determined by comparing the radioactivity remaining after various washes with that remaining after a trichloracetic acid wash. Figure 24 shows the results of such experiments. The fraction of the pool remaining is plotted against the "osmotic strength," that is, the total molar concentration of solute species in the washing solution, taking account of ionization. A relatively mild osmotic shock removes amino acid from the pool. For example, a 30% reduction in the osmotic strength will remove 40% of the pool. Washing with various concentrations of glucose removed about the same fraction as that removed with salt of the same osmotic strength. When washes are performed with 0.37 osmolar solutions of glucose, sucrose, glycine, or NaCl, the pool is unaffected. Washing with glucose solutions of higher osmolar-

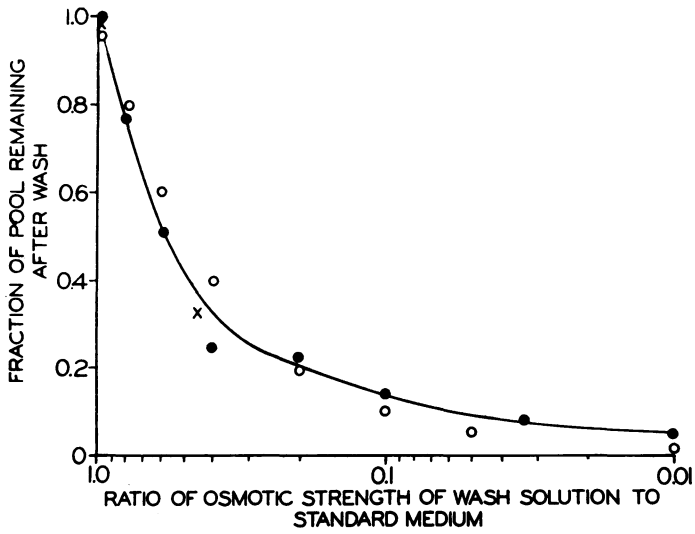

FIG. 24. *Removal of pool by washing cells with solutions of low osmotic strength. Growing cells were supplied C^{14}-proline and allowed to form a pool, and then samples were collected on a membrane filter. Various wash solutions were then passed through the cell pad on the filter. The points marked (●) were washed with various dilutions of the standard growth medium, the points marked (○) with NaCl solutions, and the points marked (×) with glucose solutions.*

ity than 0.37 has little effect on the pool. Similar results have been obtained whether the tests were carried out with large proline pools (50 μmoles per g of dry cells), small proline or valine pools (1 to 10 μmoles per g of dry cells), or on the complex pool internally synthesized from C^{14}-glucose. These results show that it is the osmotic and not the ionic strength of the washing medium that determines the fraction of the pool that is removed.

Maximum pool size as function of osmotic strength of medium. When pool formation was carried out in solutions of various osmotic strengths, it was found for small pools (< 5 μmoles per g of dry cells for a given amino acid) that the pool size was independent of the osmotic strength of the medium. However, the maximum pool size (high external amino acid concentration) is strongly dependent upon the osmotic strength of the medium. Figure 25 shows the results of a series of experiments in which the saturation pool size was measured in growth media of various osmotic strengths. The maximum pool is nearly proportional to the osmotic strength of the medium. This striking observation stands at the moment entirely without explanation.

Recovery from osmotic shock. In order to assess the type of damage caused by a sudden decrease in osmotic strength (osmotic shock), the ability of the cells to form pools and synthesize protein was measured immediately after a water wash.

A centrifuged pellet of cells from an exponentially growing culture was suspended in a small volume of water and quickly (<10 sec) added to a beaker containing the usual growth medium supplemented with C^{14}-proline. Pool formation started after a delay of less than 1 min, and protein incorporation achieved its normal value after about 1½ min. Thus, complete removal of pool compounds by the violent osmotic shock is due to a very transient change in the cell. Synthesis of any significant fraction of the cell protoplasm is not necessary in order to repair the damage.

Figure 26 shows the results of another type of experiment illustrating this same effect. While incorporation of C^{14}-proline was in process, the suspension was diluted with 2 volumes of water at the same concentration of C^{14}-proline. At the instant of dilution, the pool size dropped sharply and after 1 min started back up toward its original value.

Descriptive model of process of pool removal by osmotic shock. Speculation regarding these observations has led us to the following tentative model of the phenomenon of osmotic shock.

It is assumed that the cell contains osmotically

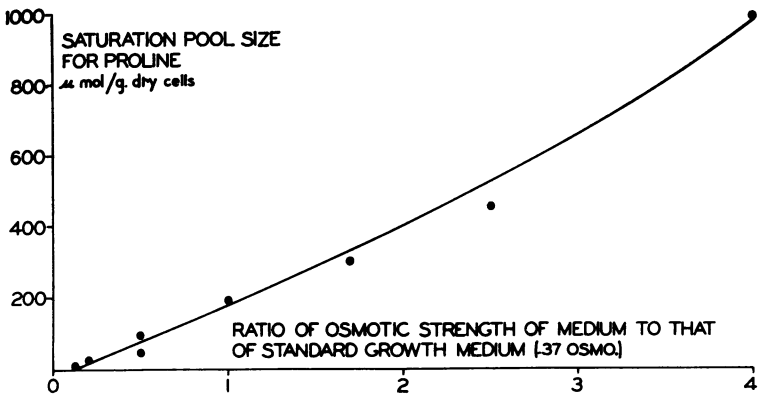

FIG. 25. *Saturation pool size as a function of the osmotic strength of the medium. The osmotic strengths shown were obtained by adding glucose to a 1:10 dilution of the usual growth medium. Growth is inhibited at the higher osmotic strengths.*

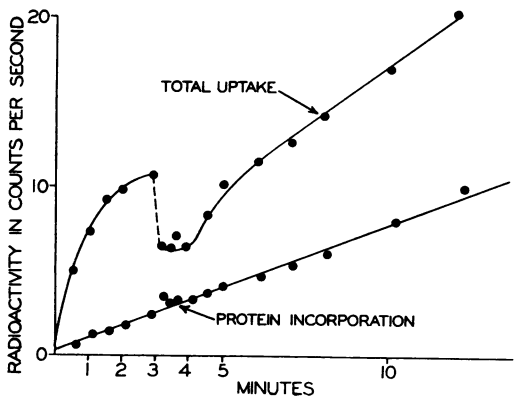

FIG. 26. *Recovery of pool, after removal by osmotic shock. At time zero, 0.3 μg per ml of C^{14}-proline was added to 0.08 mg, dry weight, per ml of growing cells. At 3 min, 2 volumes of water containing 0.3 μg per ml of C^{14}-proline were added.*

sensitive structures which are initially in osmotic equilibrium with the external medium. When the external osmotic strength is suddenly reduced, there is a flow of water into the osmotically sensitive structures along with a slow loss of solute from the cell. The consequent stretching of the structures due to the internal pressure increases the permeability to the solute, allowing a faster rate of loss of solute molecules. These two processes, together, lead finally to a new osmotic equilibrium. During this process, the cell passes through a transient state in which the structures are distended. The loss of pool is associated with this transient distention of the cell structures. The evidence concerning the osmotic sensitivity of the pool does not make it possible to decide whether the structures involved are the cell wall and membrane, structures within the cell, or a combination of these.

Pool removal versus rate of shock. Since competing rates of flow of water and solute are involved, this model suggested that a slow change in the osmotic strength might be less effective than a rapid shock. In an experiment with a large proline pool, a reduction in osmotic strength of a factor of 3 was made in four steps of equal concentration ratio. As a result, 70% of the pool was removed. If the same final osmotic strength was achieved in a single step, 90% was removed. Further, when the same final osmotic strength was achieved through a slow concentration change graded continuously over several minutes, only 50% of the pool was removed.

Uptake during shock. Another implication of this description of the process of osmotic shock is that molecules which do not ordinarily enter the osmotically sensitive structures might be able to diffuse in, during the transient period when the permeability is increased. We call this process "trick or treat," since it was suggested just after Halloween and brought to mind the children's trick of throwing in orange peels while the door is open.

This transient permeability was tested experimentally by giving a thick suspension of cells at 0 C a sudden osmotic shock in the presence of radioactive $SO_4^=$ or $PO_4^{\equiv}$. The suspension was then diluted without osmotic shock to remove diffusible label and filtered. A small amount of $SO_4^=$ or $PO_4^{\equiv}$ was taken up corresponding to

about 5% of the cell volume at the external concentration of $SO_4^{=}$ or $PO_4^{\equiv}$. Various controls showed that this uptake was indeed due to the sudden downward change in osmotic strength. Upward osmotic shocks neither remove the pool nor cause the "trick or treat" phenomenon.

This phenomenon may shed some light on earlier observations of the paradoxical conditions required for efficient production of mutations with Mn^{++} (9, 13). Effective production of mutations occurred when cells suspended in saline were transferred to a low osmotic strength medium containing Mn^{++}.

Dependence of osmotic shock on nature of solute. Studies have been made of the effectiveness of various solutes for producing or preventing osmotic shock. The results are summarized in Table 5. Since, in general, upward osmotic shock has no effect on the pool, the figures in column A simply indicate any chemical or destructive effects on the cells. Thus the compounds such as butanol remove most of the pool. The figures in column B indicate the effectiveness of the solute as an osmotic protector for short periods of time. It will be seen that, in general, higher molecular weight compounds are most effective, although the zwitterion glycine (mol wt, 75) is an effective protector, while glycerol (mol wt, 92) is not. It is presumed that solutes which do not act as protectors are able to enter the osmotically sensitive structures rapidly. The resulting excess water activity outside causes water to flow in until the distention allows internal osmotic constituents to leak out, re-establishing equilibrium.

Column C shows the effect of a sudden doubling of the osmotic strength followed rapidly by a return to the usual medium. Compounds such as acetone, which enter the cell rapidly without causing damage (as shown by the figures in columns A and B), do not cause significant removal by downward osmotic shock. On the other hand, compounds such as urea and glycerol, while still not effective protectors, are capable of removing the pool by osmotic shock.

These results indicate that, by using the removal of the pool as an index of the osmotic state within the structures, studies might be carried out on the rates of entry of a variety of small molecules.

Dependence on nature of pool. Different types of pools appear to have different sensitivity to osmotic shock. Both amino acids and phosphorus compounds (measured after labeling with $P^{32}O_4$) are completely removed by a quick water wash at room temperature. However, at 0 C, the amino acid pools are still completely removed, while only half of the phosphorus compounds are removed. Osmotic shocks (at room temperature) that remove half of the amino acid pool will remove considerably less of the phosphorus compounds. It also appears that when very large amino acid pools (formed at high osmotic strength from a casein hydrolyzate supplement) are partially removed by osmotic shock, the amino

TABLE 5. *Osmotic effects of various solutes on E. coli proline pool*

Solutes	Percentage of pool removed		
	A, upshock[a]	B, protection,[b]	C, upshock and downshock[c]
Butanol	77	100	77
Diethylene glycol	30	70	95
Ethyl acetoacetate	50	90	50
Methanol	20	100	20
Ethanol	20	100	20
Propanol	20	100	20
Acetone	10	100	15
Propionamide	10	100	20
Succinimide	20	100	20
Acetamide	20	100	50
Dioxan	5	95	40
Glycerol	5	99	70
Urea	5	97	63
NaCl	0	38	55
Na acetate	3	60	50
Diethylamine-HCl	6	35	60
Tris[d]-HCl	8	35	55
Glycine	1	16	47
Alanine	3	12	50
Valine	5	12	60
Proline	5	30	60
Glucosamine-HCl	0	5	50
Xylose	0	10	52
Glucose	0	10	52
Galactose	0	10	45
Sucrose	0	5	70

[a] Washed on filter with solute at 0.37 osmolar concentration in growth medium.

[b] Washed on filter with solute at 0.37 osmolar concentration in H_2O.

[c] As in A, followed by wash with growth medium.

[d] Tris(hydroxymethyl)aminomethane.

acid distribution in the pool is considerably altered. Thus, the amino acids in the pool show different sensitivities to removal by osmotic shock. These observations indicate that various pool materials are organized in different ways within the cell, and perhaps are associated with different substructures.

Removal of other soluble components by shock. Some studies have been made, by means of freezing point determinations, of the release of the total osmotically active material of the cell by osmotic shock. The results are broadly similar to those of studies of amino acid pools. Water washes remove the total osmotically active constituents almost completely. Boiling of the cells, after water washing, releases only traces of additional material effective in depressing the freezing point of water. When the osmotic shock is performed in small steps, the release of the total osmotically active material is quite similar to the release of amino acid pools, though perhaps a somewhat greater percentage is released for the same shock. The total quantity released from the cell indicates that if this material is osmotically active when present in the cell, the osmotic pressure within the cell is slightly greater than that of the medium and is dependent on the osmotic strength of the medium. If this material were concentrated in regions smaller than the whole cell, the osmotic pressure would be proportionately higher.

In this section, we have summarized our observations of the osmotic properties of pools in *E. coli*. While we have indicated how we picture the process responsible for the transient effects observed, the model cannot be considered unique, nor has it been developed sufficiently to test its ability to explain the results quantitatively. However, any complete model of the processes of pool formation and maintenance must be consistent with the osmotic behavior described.

L. Pools Formed in the Absence of Supplements

The "native" pool existing in a suspension of cells growing in an unsupplemented medium is the result of a balance of rates of synthesis and utilization of the amino acids. It is known that a moderate expansion of the pool by supplements will almost completely suppress the synthesis of certain amino acids (14). Thus, the native pools are probably an important link in the system that controls the rate of synthesis of low molecular weight compounds.

Estimates of the size of the native pools have been made in a few cases by adding labeled amino acid at a very low concentration. Such small quantities of amino acid are rapidly taken up into the cell, and the rate at which the label enters the protein, compared with the known rate of incorporation of the amino acid, gives a measure of the native amino acid dilution of the supplement and, by calculation, the pool size. In the cases of proline, valine, leucine, isoleucine, methionine, and tyrosine at 25 C, the label enters the protein at a rate that would lead to its exhaustion in a time between 30 and 60 sec. Thus the quantities range between 0.5 and 2 μmoles per g of dry cells for these amino acids. The label in glutamic acid, however, is exhausted only after about 10 min under these conditions, and therefore its native pool size is about 50 μmoles per g of dry cells. It does not seem worth while to quote the precise results of these measurements, since they are quite variable, and no detailed studies of the causes of variation have been carried out. Native pools for valine have been observed as large as 15 μmoles per g of dry cells, although they are normally one-tenth of this size.

These native pools normally exist in equilibrium with a quantity of amino acid released to the medium. Measurements of the internal concentration relative to that present externally in unsupplemented cultures have not been carried out for growing cells. However, the fate of a very small quantity of glucose (described in the section on energy requirement) shows that the concentration ratios are very high for some amino acids in the absence of glucose. The figure for valine is 28,000:1, in the absence of glucose. It is greater than 18,000:1 in the presence of glucose, as shown by the experiment of Fig. 21.

M. Miscellaneous Observations Related to the Pool

In the studies of the pool formation process, a number of incidental observations have been made which do not at the moment lead to any definite conclusions. Nevertheless, it seems worthwhile to put them on the record in this section.

Since chloramphenicol is thought to suppress specifically protein synthesis, it might seem to be a useful tool for the study of pool formation. It would be expected to increase the pool somewhat

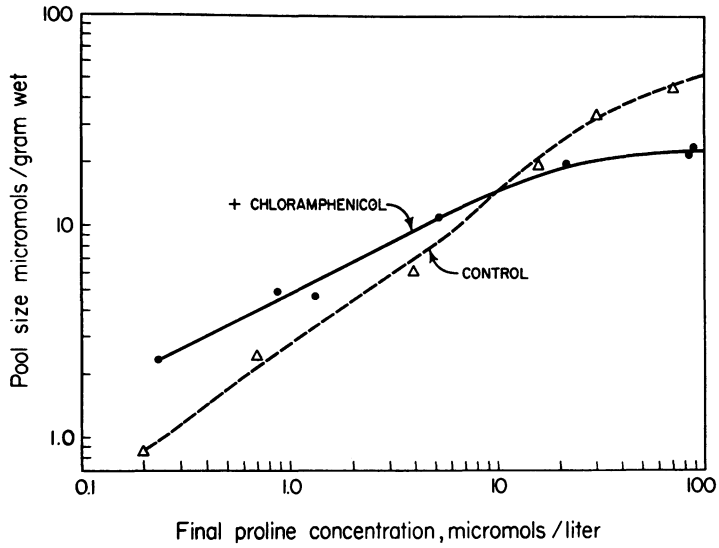

FIG. 27. *Effect of chloramphenicol on the proline pool. Chloramphenicol, 20 μg per ml, was added to a growing culture of cells at 25 C. Five minutes later the appropriate concentration of C^{14}-proline was added. The kinetics of pool formation were followed, and the pool size and external concentration were estimated after the pool had leveled off at its maximum value. The results are shown by the solid circles and solid line. The open triangles and dashed line show the pool size for growing cells from Fig. 20.*

since the drain from the pool into protein would not be present. The observations, however, show that it interferes in some way with the formation of large pools. Figure 27 shows the results of a study of the proline pool size as a function of concentration in the presence of 20 μg per ml of chloramphenicol. For comparison the pool size in growing cells is shown (△) by the dashed line. It appears that the maximum pool size is reduced by a factor of more than 2 by chloramphenicol. An increase in the pool size at low concentrations is observed as expected from the removal of the drain from the pool into protein.

In magnesium-deficient media, the ability to form amino acid pools in *E. coli* was markedly reduced, as was the rate of incorporation into the trichloroacetic acid-precipitable fraction. Reductions of as much as a factor of 5 in pool size have been observed, but the results are quite variable, presumably as a result of uncontrolled traces of Mg present in the "Mg-deficient" media.

When *E. coli* cells are exposed for a few minutes to a hydrostatic pressure of 20,000 psi, a large part of the amino acid pool is released to the medium. Even though growth of the cells is inhibited for an hour or so after the pressure is removed, most of the released pool is quickly reincorporated. The sensitivity of the pool to hydrostatic pressure presumably results from the distortion of the structures holding the amino acid pool, and probably is related to the sensitivity of the pool to osmotic shock.

Some exploratory studies have been made on the reaction of hydroxylamine with the pool amino acids of *E. coli*. Hydroxylamine reacts rapidly with acyl phosphates, anhydrides, and halides to form hydroxamic acids; the corresponding carboxylates, amides, and peptides react very slowly. If the pool amino acids were present as activated forms such as acyl phosphates, a fairly efficient conversion to the corresponding amino hydroxamic acids would be expected in the presence of high concentrations of hydroxylamine. Experiments to test this possibility show very small yields of amino hydroxamic acids. In the two cases tested with great sensitivity, identifiable quantities of leucine and tyrosine hydroxamic acids have been observed. These quantities, however, correspond to a very small fraction of the native pool of these amino acids. In fact they were not present in an amount sufficient to supply the cells' requirement for protein synthesis for as long as 1 sec. The

experiments on the synthesis of nascent protein (12), however, indicate that the whole process from external sulfate to amino acid to protein is completed in about 3 sec. Thus the fact that activated amino acids are present in extremely small quantity does not, by itself, indicate whether or not they play a role in protein synthesis in the growing bacterial cell.

III. Discussion of the Mechanism of Pool Formation

A. Introduction

The purpose of this section is to examine the implications of the large number of experimental observations presented in the previous sections. It appears necessary, in order to bring some clarity to a problem of this complexity, to start out by postulating models of the process. The discussion of the experimental observations in relation to the models allows the implications to be brought out more clearly.

The qualitative predictions of the relatively simple models considered here can be deduced easily. However, our knowledge of their quantitative predictions must depend on a fairly crude analysis. What are undoubtedly complex reaction sequences are taken to be single steps subject to the simpler equations of chemical kinetics. The coupling of the concentration process to the cell's energy supply, for example, is included in an almost purely symbolic way.

The state of the subject at this writing is that the two simple models that have been widely discussed, termed by Monod *permease* and *stoichiometric site*, both fail in a qualitative way to explain the presently known facts. Following a discussion of the reasons for these failures, a new model is proposed, which we have called the *carrier model*. The deductions from this model are consistent with practically all of the observations.

B. The Permease Model

The permease model is shown schematically in Fig. 28. Cohen and Monod state (8), "Obviously the actual mechanism of specific permeation must be more complex than the deliberately bare and abstract model we have set up." Further, they use the term "permease" nearly synonymously with "specific permeation mechanism." However, for reasons of clarity, in this discussion the term "permease model" will be restricted to a model with the essential features listed in Fig. 28. The list has been abstracted from (8) and is written for the case of an energy-dependent amino acid-concentrating system.

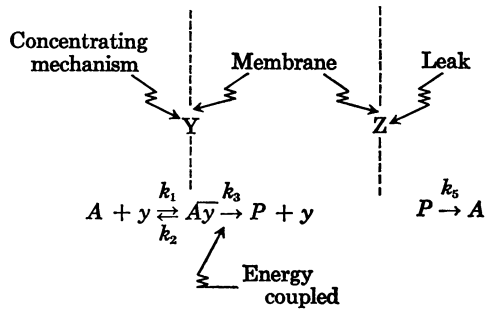

FIG. 28. *Permease model. Properties:*
a) The bacterial cell is enclosed with an osmotic barrier which is highly impermeable to amino acids;
b) The impermeability is not absolute and leakage may occur, tending slowly to equilibrate the inside and outside concentrations;
c) Within the barrier exist proteins (the permease) capable of forming specific complexes with the amino acid;
d) The complex associates and dissociates reversibly with amino acid either inside or outside the barrier, and catalytically activates the equilibration of internal and external concentrations;
e) When coupled to an energy donor, the internal association reaction is, in effect, inhibited, and amino acid accumulates inside the cell;
f) As the internal concentration rises, the nonspecific leakage increases until its rate balances the rate of accumulation at equilibrium;
g) The amino acid is presumed to be in a free state within the cell.

The permease model accounts for a great many of the observations regarding the concentration of substances by bacteria. There is no need here to repeat the extensive review of the evidence given in (8). It is sufficient for the moment to say that the evidence clearly shows that there are stereospecific sites which act as catalysts for the concentration of compounds by the cell. In other words, there are sites which participate in the early steps of the process but do not, by themselves, hold the compounds in the cell. Also some evidence indicates that the catalytic sites are protein in nature. Such sites will certainly have to be retained as a part of any adequate model for the pool-forming process.

TABLE 6. *Relationship of properties of the pool to the models*

Observation	Score[a]	
	Permease model	Carrier model
Formation and maintenance:		
1. Glucose required for formation	+	+
2. Glucose not required for maintenance	−	+
3. Pools formed slowly at 0 C	−	+
4. Pools maintained at 0 C	−	+
5. Pool maintained at 25 C in absence of either the amino acid or glucose	−	+
6. Pool size versus concentration not Michaelis	−	+
7. Initial rate of formation not proportional to pool size	−	+
8. Small pools not generally influenced by other amino acids but large pools are suppressed	−	+
9. Evidence for catalytic site in general	+	+
10. Pools may be very large	+	−
Exchange:		
11. Exchange occurs in addition to steady flow through the pool	+	+
12. Rapid exchange occurs in absence of glucose or at 0 C	−	+
13. Fast and slow components in exchange at 0 C	−	+
14. The 0 C exchange rate saturates at low external concentration	−	+
15. The 0 C exchange rate increases with pool size	−	+
Osmotic behavior:		
16. Pools removed by sudden reduction in osmotic strength	+	(+)[b]
17. Pools immediately re-formed after removal by shock	+	+
18. Different pools removed to different extent by shock	−	(+)[b]
19. Maximum pool size increases with osmotic strength of medium	−	−

[a] A + sign indicates that the model satisfactorily explains the observation. A − sign indicates that there is a contradiction or that a modification may be required by the experimental evidence.

[b] Assuming that the sites may be osmotically sensitive.

The permease model predicts a number of features of the concentration process which are contradicted by the evidence. In order to visualize these contradictions, we will first consider the way in which a pool is formed according to the permease model, and then discuss the experimental observations listed in Table 6.

An external amino acid first forms a complex with the permease (y) in the osmotic barrier. In the presence of an energy supply, the complex dissociates on the inside of the barrier. The free amino acid within the cell may then pass out of the cell by means of a nonspecific leak (z). Thus there will be a continuous circulation of amino acid through the pool at equilibrium when the two rates balance. For a given external concentration, the pool size will be determined by the rate at which the permease can pump amino acid into the cell. At the external concentration at which this rate saturates, the pool size will saturate.

The observations (Table 6, items 1 and 2) that glucose is not required to maintain a large internal concentration but is required to form a pool at normal rates clearly are not consistent with the above description of the pool-forming process. Similarly, the permease model fails to explain the observation (items 3 and 4) that, while pools are formed very slowly at 0 C, pools formed before chilling are maintained indefinitely (if not too large a size). From the point of view of the permease model, these results imply that when glucose is exhausted or the temperature is reduced to 0 C, not only is the active permeation process suppressed, but the "passive" leak is also suppressed to an *identical degree*. Further, the pool is maintained at 25 C in the absence of the amino acid, whether or not glucose is present. Thus, item d in the list of properties of the permease model is contradicted.

These observations clearly establish that a passive leak, as measured by pool maintenance

experiments, is very much too small to control the steady-state pool size by balancing against a simple active input process. They imply either that the leak must be made a rather strange function of the environmental conditions or that a more sophisticated active process must be considered. As will be illustrated in the discussion of the carrier model, a self-suppressing input mechanism seems to provide a more direct and satisfactory explanation of the empirical data.

The evidence on the exchange between external and pool amino acids summarized in items 12, 13, and 14 of Table 6 raises further difficulties for the model. Exchange under normal conditions, for example at 25 C, is expected, owing to the circulation through the pump and leak. The measured rate of exchange is comparable to the initial formation rate, as the model predicts. However, the high rates of exchange in the absence of glucose or at 0 C are not predicted. It might be suggested that the complex $\overline{Ay}$ can exchange with both A and P, at a rate higher than the actual association or dissociation in the absence of energy. The fact that the rate of exchange (at 0 C) saturates at a low concentration would suggest that the permease itself is saturated. In addition, some property of the permease must limit the rate of exchange. This is perhaps reasonable, since the complex cannot be exposed on the inside and outside of the membrane at the same moment. The permease model has been given a minus score in Table 6 for items 12, 13, 14, and 15 since an exchange mechanism must be added. In fact, this mechanism would have to be specified in detail in order to make an adequate comparison with the observations.

Item 12 shows clearly that the pool has more than one component. This argument has been given in detail in the experimental section on exchange. The evidence summarized in items 6, 8, and 18 also strongly supports this conclusion. From the point of view of the permease model, one might say that the cell has several pool-containing compartments separated by osmotic barriers, each containing an appropriate permease, but this is not a pleasant prospect. It seems much more likely that at least part of the pool is not in free solution within the cell, as will be discussed below.

The observation (item 6) that the pool size, far below saturation, does not rise in proportion to the concentration could be taken to indicate that there is more than one component in the pool or that there are permeases with a variety of affinities for a given compound. For a number of reasons, this item cannot be considered a crucial argument against the permease concept. However, since the permease model we are discussing does not predict such a result, it is given a minus score for item 6.

The initial rate of pool formation (item 7) at the time the amino acid is supplied shows a much smaller variation with concentration than does the pool size. In fact, it saturates at a relatively low concentration. A similar conclusion can be drawn from the observation that a low concentration of isoleucine will block valine incorporation (Fig. 7). Both these observations show that the catalytic site for pool formation saturates at a concentration far below that at which the pool itself saturates. For this reason, these observations supply good evidence for the existence of a catalytic site. A characteristic of the permease model is that the circulating flow is proportional to the pool size. This circulating flow is, in turn, identical with the initial rate of pool formation. The failure of this proportionality further supports the conclusion that the pool is maintained by mechanisms other than the balance between a rapid active process and corresponding rapid leak.

It is clear from the above discussion that the simple permease model is inadequate because it fails to agree with experimental data in a number of ways. In addition, there is a philosophical objection to the model as written. This may be seen by noting that P is the same material as A, except that it is on the other side of the barrier. Why, then, don't P and y interact with the same rate constants (k_1 and k_2) as A and y? Of course, the energy-coupled reaction (k_3) may occur only inside the cell because the energy carrier may be so localized. Now it might be suggested that if the energy-coupled reaction (k_3) is fast enough it will be so dominant that the ordinary reactions (k_1 and k_2) could be neglected inside the cell. However, note that the input rate is not greater than k_1yA. Therefore $k_3\overline{Ay}$ is also not greater than k_1yA. But k_1yP is much greater than k_1yA simply because P is ordinarily much greater than A. Thus it is seen that this philosophical objection is hardly pedantic, and one in fact has assumed the existence

of some rather tricky means of distinguishing the performances inside and outside the cell. This mechanism really is the key to producing the desired behavior, and the failure to display it explicitly simply avoids the whole question. In this sense the model really is not a scientific model at all. If we are to say that the materials inside the cell are somehow physically or chemically different from those outside, doesn't the real elucidation of the problem lie in explaining the nature of this difference?

From the experimental side, it is again worth noting that, to explain the relationship between pool sizes, loss rates, and initial formation rates, it would appear necessary to postulate a mechanism whose details provide that the size of the pool is not determined solely by the loss rate increasing until it equals the input, but rather by a heavy contribution from the input rate being lowered as the pool increases in size. In other words, the pool should inhibit its own formation.

One can postulate detailed mechanisms which meet both the philosophical objection to the simple model and the inhibiting requirement mentioned above. With such improvements the permease model becomes more satisfactory, but in order to make it fit the full variety of the experimental facts, even greater complexity and sophistication are apparently required. It seems out of place in this review to illustrate the possibilities of a number of models of increasing complexity. Suffice it to say that it may be possible to add enough special features to make a reasonably satisfactory model in which the elements of the simple permease model may perhaps still be recognized. In particular, the crucial part played by the osmotic barrier would presumably still be dominant in such an extended model.

C. The Stoichiometric Site Model

The central feature of any "site" model is that the amino acid is held by association with the macromolecules of the cell. For the moment, the nature of this association is unspecified, and it is presumed that the osmotic barrier which may exist near the surface of the cell is of minor importance in the maintenance of the pool. The simplest possible model of this type is shown schematically in Fig. 29.

It is immediately obvious that this model is unsatisfactory, since it does not explicitly contain a step equivalent to the catalytic site. Further, the rate of pool formation should be proportional to the amino acid concentration and the number of unoccupied sites.

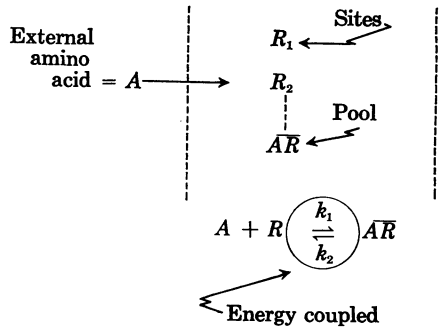

FIG. 29. *Stoichiometric site model*

In order that the pool size increase with the external concentration (at low concentrations), there must be a process by which the site-amino acid complexes dissociate. The fraction occupied would then be determined by a balance between the rate of formation and dissociation. The rate of dissociation, however, would have to be energy-dependent, in order that the pool be maintained as implied by items 2, 3, 4, and 5. Since all of these difficulties can be resolved together, we shall pass immediately to the discussion of the "carrier" model.

D. The Carrier Model

The carrier model is consistent with practically all of the known facts concerning amino acid pools. We have been led to postulate its central features by the failure of the previously discussed models.

The fact that pools are maintained under adverse conditions where they might be expected to leak out, combined with other evidence, has led us to include sites as the major mechanism for maintaining the pool. The strong evidence that a catalytic site participates in pool formation has led us to include such an intermediate step in the formation of the site-amino acid complex (pool). We have, therefore, postulated that the catalytic site is part of a molecule of moderate molecular weight termed the "carrier." The carrier molecule is assumed to be large enough to form a stereospecific complex with the amino acid, but still small enough to diffuse within the cell with some freedom. The mobility of the carrier is necessary, since there are few carriers

to transfer amino acids to the many pool-holding sites.

A schematic diagram of the carrier model is shown in Fig. 30, along with equations indicating the reactions proposed. The listed properties of the model have been specified quite sharply so that the deductions may be quantitatively analyzed. It cannot be ruled out that certain "forbidden" processes, such as exchange between free amino acid and site-associated amino acids, proceed at slow rates. Further, the evidence is insufficient to specify which of the two processes is actually coupled to an energy donor.

According to this model, the pool is formed in the following way. An external amino acid diffuses into the cell and collides with an unoccupied carrier. A complex with the stereospecific carrier is formed ($\overline{AE}$); the complex diffuses through the cell and collides with an unoccupied site. In a reaction coupled to an energy donor, the amino acid is transferred from the carrier to the site. In turn, an unoccupied carrier may collide with an occupied site and remove the amino acid. The evidence at the moment does not specify whether or not the reverse reaction is also energy-requiring.

As the pool rises, the reverse reaction ($\overline{AR} + E \rightarrow \overline{AE} + R$) reduces the quantity of free carrier (E). Thus, the rate of formation of carrier complex with free amino acid falls until it equals the rate required for protein synthesis. This is then the steady state.

With reference again to Table 6, the deductions from this model will now be compared with the observations. Item 1 is obvious, since the energy requirement has been built in. Two distinct properties of the model lead to maintenance of the pool when formation is suppressed (items 2, 3, 4, and 5). We could assume the reverse reaction ($\overline{AR} + E \rightarrow \overline{AE} + R$) to be energy-dependent, and then clearly the pool would be maintained in the absence of glucose. Alternatively, we are at liberty to choose a small value for the constant, k_2, without influencing any other properties of the model, and therefore the loss rate under all conditions (short of damaging the cell) can be set as low as necessary. The choice of a small k_2 simply means that the carrier (or catalytic site) has a high affinity for the amino acid, and thus the amount of carrier complex will saturate at low concentrations. This is consistent with the saturation of the exchange rate (item 14) and of the formation rate (item 7) at low external concentrations.

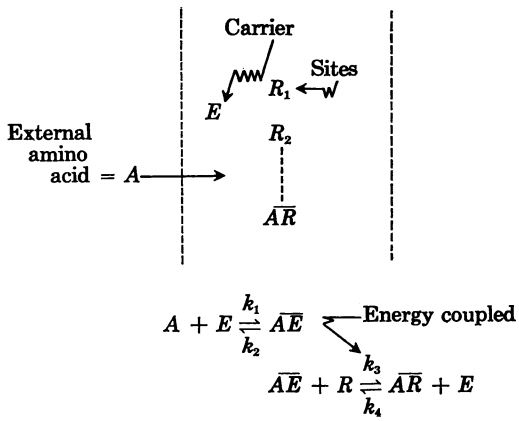

FIG. 30. *Carrier model. Properties:*

a) The cell contains a small quantity of mobile stereospecific carriers which freely form complexes ($\overline{AE}$) with amino acids, without participation of energy donors;

b) The cell also contains a relatively large quantity of nonmobile groups (the sites) which form complexes ($\overline{AR}$) with the amino acids;

c) The site complex $\overline{AR}$ can only be formed by a reaction with the carrier-complex $\overline{AE}$ and this reaction is coupled to an energy donor;

d) Exchange may occur between the site-associated amino acids and those associated with carriers, without coupling to energy donors;

e) Exchange also occurs between free amino acids and carrier-associated amino acids, but not between free amino acids and site-associated amino acids;

f) There are several classes of sites, some stereospecific and some nonspecific;

g) There may be an osmotic barrier near the surface of the cell and "free" amino acid may not diffuse through the protoplasm at the same rate as in water, but the formation of the carrier complex nevertheless occurs at a sufficient rate, without the participation of an energy donor.

The evidence on exchange between pool and external amino acids led to the postulation of the exchange processes described in items d and e in Fig. 30. (In this connection one might consider the discussion at the end of Part I-H). These processes are sufficient to explain all the observations on exchange (items 11, 12, 13, 14, and 15 of Table 6). Saturation of the exchange rate at low external concentrations is predicted if the natural assumption is made that the exchange rate between free amino acid and carrier

is more rapid than the exchange rate between the amino acids of the carrier complex and the site complex. Thus, when exchange is studied with labeled amino acid, the specific radioactivity of the amino acid associated with the carrier complex would always be close to that of the external amino acid. The amount of the carrier complex is saturated under the conditions of the experiment at 0 C. The collisions between the carrier complexes (constant specific activity and quantity) and the site complexes would control the exchange. The rate of exchange would, therefore, be independent of the external concentration and proportional to the pool size.

In the mathematical appendix, the carrier model is examined in some detail. Allowance has been made for two different kinds of sites. In this model it is important to account for the utilization of amino acid for protein synthesis, for the native pool, and for the competition between internally synthesized and exogenous amino acid if quantitative evaluation of the model is to be made. The constants of the model (for proline in *E. coli*) which give a relatively good quantitative correlation with the data are presented in the appendix. These constants were obtained from experiments on pool sizes and rates of formation. It is very pleasing that so much information on the details of the concentration relationships and the competition between synthetic processes and concentrating processes can be encompassed in one conceptually simple model. One interesting consequence of the treatment is that the two sets of sites correspond to two components of the pool, one of which has a saturation value 20 times the other. The larger component has a half-saturation value of external concentration which is 100 times that of the smaller and, as a consequence, is only dominant at the higher end of the concentration range. It is very satisfying that the existence of two components of the pool of such different characteristics provides qualitative agreement with conclusions deriving from experiments on exchange and osmotic shock.

The removal of the pool by osmotic shock (items 16, 17, and 18) is not an obvious prediction of the carrier model. The simplest explanation, from the point of view of this model, is that the sites themselves are temporarily affected during a transient period of distention of the cell. The implication is that the macromolecules on which the sites are located are temporarily distorted in such a way that the affinity of the site for amino acid is drastically reduced. This could result from a direct change in the hydration of the macromolecule itself or from a mechanical coupling of the macromolecule to major cell structures. It must be pointed out that a semipermeable membrane is not necessary for osmotic phenomena to occur. An ion exchange column (Dowex 50, 2% cross-linked) will undergo striking volume changes when sucrose solutions of different concentrations are passed over it.

From this point of view, some interesting speculation about items 19 and 10 may be indulged in. The size of a very large pool, which is nonspecific, is roughly proportional to the osmotic strength of the medium. Thus, the maximum pool is somehow related to the osmotic balance of the cell, although even the largest amino acid pools account for only a small fraction of the total osmotically active material that may be released from the cell. It may be suggested that there exist nonspecific associations between the amino acids and the dense protoplasm (25% dry material) of the bacterial cell. The maximum quantity of amino acid in such an association might decrease with increasing hydration of the protoplasm. No such associations are observed in relatively dilute protein solutions or in disrupted suspensions of cells. However, a carrier or energy donor present in the living cell might be a necessary condition in the formation of such an association.

The fact that different pool compounds are removed in varying degrees by a given osmotic shock (item 18) probably reflects the differences in sensitivity of site complexes.

Finally, it is difficult to leave the discussion of this model without some speculation on the nature of the carrier. The properties required of the carrier, for its function in this model, are that it be a large enough molecule to form a stereospecific association with an amino acid and, on the other hand, that it be small enough to diffuse with some freedom within the cell. Further, it must have a high affinity for free amino acid and be able to give up amino acid freely to form a site complex. In turn, unoccupied carriers must be able to accept amino acids from the site complexes.

At least two possible candidates for the carrier are known at present. The lipid-amino acid

complex discovered by Hendler (10) in the hen oviduct has been observed in *E. coli*. The quantity and rapidity of labeling of such complexes in tracer experiments are consistent with the possibility of their function as carriers in the sense of this model. However, the molecular weight is unknown. The soluble ribonucleic acid amino acid complex discovered by Hoagland also occurs in *E. coli* in very small quantities. However, there is no indication (1) that the rate of turnover of the amino acid in this complex is fast enough to carry out the function of the carrier.

A crude lower limit on the amount of carrier present in the cell can be set from its turnover number and rate of diffusion. At the maximum rate of proline pool formation, there are 40,000 molecules entering the pool per sec per cell. The time required for a small molecule such as proline to diffuse 1 μ is about 1 msec. The mean distance over which the carrier must diffuse between taking up an amino acid and delivering it to a site is much smaller than this. However, the molecular weight is probably much larger, and the diffusion constant is probably much smaller, in the protoplasm than in water. Thus, the turnover number probably would be considerably less than 1,000 per carrier per sec, and therefore the number of carriers would be greater than 40 per cell. If the number of carriers were as small as this, it would certainly be difficult to observe the carrier complex directly.

The properties of "cryptic" mutants have had an important place in discussion of bacterial-concentrating mechanisms (7). They have not been mentioned so far, since we have limited ourselves to amino acid-concentrating systems. For our purposes, the observations may be summarized as follows. There are strains of *E. coli* (y^-, z^+, i^-) which contain large quantities of the enzyme, β-galactosidase, but will not utilize lactose or concentrate galactosides. The enzyme is fully active in preparations of these cells treated with toluene. Undamaged cells will only split the test-substrate (*o*-nitrophenylgalactoside, ONPG) at low rates when it is supplied at high concentrations. Other strains which utilize lactose contain both the enzyme and an operative concentrating system. The enzyme in these cells will split ONPG at high rates whether treated with toluene or not and when the concentration of galactosides has been blocked by metabolic inhibitors.

The fact that the mechanism for the concentration of galactosides and the enzyme, β-galactosidase, are controlled by distinct genetic loci is consistent with the carrier model. Clearly the carrier, the sites, and the enzyme would be three distinct elements in the cell. However, in the carrier model no osmotic barrier limits the rate of entry of substrate. What, then, limits the rate of splitting of the substrate (ONPG) by the enzyme present in the cryptic mutant (y^-, z^+, i^-)? An additional hypothesis is necessary: In the organized cell, the free substrate does not have full access to the enzyme, but when associated with the carrier, it can reach the active site and be attacked at maximal rate. In defense of this hypothesis, it may be pointed out that there are a large number of examples of enzymatic reactions which are suppressed or absent in whole cells but occur at high rates in disrupted cell preparations. In some cases, the suppression has been attributed to an impermeable barrier, but there are a number of examples for which the substrate is known to be present in the cell, and such an explanation is clearly invalid. The fact that (in y^+ strains) metabolic inhibitors block the concentration process but do not reduce the rate of splitting of ONPG further implies that the carrier complex is formed rapidly without the participation of energy donors.

Support for the carrier model also comes from experiments on the concentration and utilization of nucleic acid bases by *E. coli* (11; M. Buchwald and R. J. Britten, *unpublished data*). The experimental facts, however, differ in almost every conceivable way from those relating to amino acid concentration processes.

The bases are almost instantaneously converted to nucleotides. The rate of incorporation of an exogenous base into ribonucleic acid (RNA) may reach, within 5 sec, half the total rate of incorporation of the corresponding residue into RNA. Thus there exists a "by-pass" around the large pool of nucleotides stored in the cell. Further, the size of the nucleotide pool is independent of the external concentration of the base; i.e., the nucleotide pools are not expandable. The maximum rate of uptake of the base by the cell is not larger than the requirement for RNA synthesis. Nevertheless the cells rapidly take up bases at low concentrations (10^{-7} M).

It appears that simply by changing the values of the reaction rate constants, the predictions of the carrier model may be altered to agree with the observations of the incorporation of bases into nucleic acid. Specifically, if k_3 is very much greater than k_4 (Fig. 30), then there will be few free sites (R), and the pool will always be near the saturation level. Further, the small quantity of free sites reduces the rate of transfer of the nucleotide from the carrier complex to the storage sites. Thus the rate of entry into RNA may be comparable and the "by-pass" is explained. Of course, if the pool is always near saturation, the rate of entry into the cell cannot be greater than the rate of incorporation into RNA.

Quantitative evaluation of the rate constants for the incorporation of nucleic acid bases has not yet been carried out; it appears obvious, however, that the carrier model is capable of explaining qualitatively all of the known features of the process. These features would be quite difficult if not impossible to interpret, if it were assumed that the nucleotides were in free solution within the cell. The very existence of the by-pass demonstrates that at least a part of the pool is organized in the cell in a very different way from the large pool of nucleotides.

E. Conclusion

In this paper we have outlined the rather diverse set of experimental observations of the amino-acid-concentrating processes in *E. coli*. Any satisfactory model of these processes must be formulated with all of this information in mind.

We have reviewed the simple "permease" and simple "stoichiometric site" models and pointed out their failures. Then we have developed the "carrier" model in some detail and demonstrated that it can be made to correlate almost all of the data in a highly satisfying manner.

While it is tempting to do so, it should not be asserted that the function of sites and carriers has been rigorously proved or that the function of an osmotic barrier has been demonstrated to be unimportant. All that can be said is that the equations derived from the carrier model accurately describe the experimental data. Possibly these equations are not unique to that model. Any model which gave essentially the same equations would also be satisfactory, and one which gave essentially different equations, clearly unsatisfactory.

The permease model might be elaborated to remove some of its difficulties. Part of such an elaboration would have to consist of specifying the mechanism by which asymmetry in reaction constants on the two sides of the barrier is obtained. This mechanism might introduce additional features which would modify greatly the properties of the permease model. We must ask whether any mechanism can be proposed which has the necessary properties, if the pool is assumed to be free in solution. The minimal requirements are rapid pool formation; slow loss in absence of glucose, or amino acid, or both; increase of pool with external concentration until saturation is reached; and lack of proportionality between formation rate and pool size. If an exhaustive search does not yield a model with these properties, then there would be no reasonable alternative but to assume that the amino acid pool is not in free solution within the cell. In this connection it should be remembered that experimental data conclusively demonstrate that the pool has at least two components, so that if we are to assume that the pool is unbound amino acid, we must introduce at least one more osmotic barrier. Possibly it would be necessary to resort to a combination of a site and barrier.

Of course, there are many problems which deserve further investigation. The meaning of the term "free in solution" needs to be examined both experimentally and theoretically. A 25% solution of protein, RNA, etc., organized in subtle ways, is certainly an unusual solvent from a chemical point of view. The activity of the amino acids might be strikingly depressed. The results of such a study would have very broad implications for other processes in living cells.

Finally, a more detailed experimental study of the rates of loss and exchange is needed. The study of these phenomena with pools of several amino acids and other compounds, such as galactosides, would supply quantitative information that might be helpful in deciding among the alternative mechanisms.

With present knowledge, alternative interpretations of the concentration process in bacteria still remain possible, in spite of the large amount of experimental evidence which has given insight into many aspects of the process. The simplicity and the degree to which individual steps may be understood, from a chemical point of view, differ among the various

models. It is for the future to decide which of the alternative approaches will be most useful.

IV. Appendix: Mathematical Analysis of the Models

A. Method of Calculation

For the purposes of making these chemical kinetic calculations, it has been presumed that the main steps postulated for each of the models behave as simple molecular reactions. In other words, in the reaction, $A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \overline{AR}$, the rate of formation of the complex is simply k_1AR, and the rate of dissociation is $k_2\overline{AR}$. The possibility of intermediate steps or other complicating factors has been ignored. Such an approach can only be defended by its usefulness in supplying insight into the proposed models and by the agreement of the results of the calculations with experimental data.

In much of the analysis which follows it has been presumed that the external amino acid concentration is constant throughout the period in which the pool is built. In other words, of the total amino acid present in the system, only a small fraction is taken up into the pool. This situation is easily achieved experimentally at low cell densities. Correction must be made for changing concentrations in the evaluation of experiments at higher cell densities.

The system being examined in this analysis is bounded by the external surface of the cell, and all of the internal concentrations are expressed per unit volume of wet cells. When the external concentration remains constant, the volume of medium surrounding the cells does not enter the equations. In general, the calculation should be restricted to a region, of unknown volume, smaller than the whole cell. If it were possible to define the boundaries of the system to coincide with the actual reaction volume, the effective internal concentrations would be higher. Thus, some of the derived rate constants are subject to this uncertainty as far as their absolute values are concerned.

For simplicity, the utilization of pool amino acid for the synthesis of protein has not been taken into account in the analysis of the permease model. This approximation is justified for this model, since there is always a large flux into and out of the pool through the permease and leak. At high concentrations the flux is 10 times the rate of utilization for protein, and at the lower concentrations studied the flux is 3 times the protein rate. Thus, at low concentrations, the predicted pool values would be lowered by taking the protein rate into account. Even without this correction, however, the predicted values fall well below the measured values. For the carrier model, on the other hand, the situation is quite different. In this case the rate of flow of amino acid into the pool is strongly suppressed as the pool rises toward its steady value, and therefore the flow into protein cannot be ignored.

The following symbols have been used to represent the concentration per unit cell volume of the various species entering the reactions:

y = free permease
$\overline{Ay}$ = permease-amino acid complex
$Q = y + \overline{Ay}$ = total permease
P = pool amino acid
E = free carrier
$\overline{AE}$ = carrier-amino acid complex
$z = E + \overline{AE}$ = total carrier
R = free site
$\overline{AR}$ = site-amino acid complex (pool)
$\pi = R + \overline{AR}$ = total sites of one class
ρ = volume fraction in cells.

In addition, A represents the external amino acid concentration per unit volume of medium; k_1, k_2, etc. represent the rate constants for the various reactions; and α represents the constant rate of utilization of pool amino acid for the synthesis of protein.

B. The Permease Model

The reactions postulated for the permease model are shown in Fig. 28. The rate of change of the amount of permease complex is simply the sum of the rates of formation and dissociation in the three reactions indicated by k_1, k_2, and k_3.

$$\frac{d\overline{Ay}}{dt} = k_1 Ay - k_2\overline{Ay} - k_3\overline{Ay} \qquad (1)$$

Similarly, the rate of change of the pool can be written as

$$\frac{dP}{dt} = k_3\overline{Ay} - k_5 P \qquad (2)$$

The total quantity of permease is

$$Q = \overline{Ay} + y \qquad (3)$$

When the pool has reached a steady value, both

equations 1 and 2 are equal to zero. Therefore, from equations 1 and 3,

$$\overline{Ay} = Q \frac{A}{\frac{k_2 + k_3}{k_1} + A} \tag{4}$$

Substituting this result into equation 2,

$$P = \frac{k_3 Q}{k_5} \cdot \frac{A}{\frac{k_2 + k_3}{k_1} + A} \tag{5}$$

Thus the pool size, as expected, follows a classical adsorption isotherm.

The initial rate of formation of the pool is most easily evaluated by making the so-called steady-state approximation. The amount of permease complex changes slowly or not at all after it almost instantaneously reaches its steady value. Thus, equation 1 may be taken to be equal to zero; therefore, equation 4 again results. Equation 4 is substituted in equation 2, taking the leak rate, k_5P, to be negligible at early times (P is small).

$$\left(\frac{dP}{dt}\right)_{P=0} = k_3 \overline{Ay} = k_3 Q \frac{A}{\frac{k_2 + k_3}{k_1} + A} \tag{6}$$

Thus, by comparing equations 5 and 6, it is seen that the initial rate of formation is proportional to the pool size that is finally achieved for systems of low cell density.

The initial rate of loss from the pool when the external amino acid is removed also is easily evaluated. Since in this model the permease complex, $\overline{Ay}$, can only be formed from outside, $\overline{Ay} = 0$ when $A = 0$. Therefore,

$$\left(\frac{dP}{dt}\right)_{A=0} = -k_5 P \tag{7}$$

Thus, by comparing equations 5, 6, and 7, it is clear that the initial rate of loss after the amino acid is removed is identical with the initial rate of formation when the amino acid was originally added for systems of low cell density.

C. The Stoichiometric Site Model

The reaction for this simple model is

$$A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \overline{AR}$$

Also,

$$R + \overline{AR} = \pi \tag{1}$$

The rate of change of the pool can be written as

$$\frac{d\overline{AR}}{dt} = k_1 AR - k_2 AR \tag{2}$$

When a steady pool is achieved, equation 2 equals zero; by using equation 1,

$$\overline{AR} = \pi \frac{A}{A + k_2/k_1} \tag{3}$$

The rate of formation when the pool is zero, $(d\overline{AR}/dt)_{\overline{AR}=0} = k_1 A\pi$, does not saturate with increasing external concentration.

The rate of loss when $A = 0$ is

$$\left(\frac{d\overline{AR}}{dt}\right)_{A=0} = -k_2 \overline{AR}$$

Therefore, the rate of loss is proportional to the pool size. Using equation 3, we see that, at small external concentrations, the rate of loss equals the initial rate of formation. At large concentrations it is smaller.

D. The Carrier Model

The reactions postulated for this model are shown in Fig. 30. The experimental evidence has shown that loss from the pool occurs only at low rates, and this fact is reflected in the model. As a result, when a steady pool is formed (in growing cells), the rate of uptake into the cell is approximately balanced by the rate of utilization for protein synthesis. Thus, the utilization for protein synthesis must be taken into account. Further, when the external concentration is small, the cell synthesizes a significant part of its amino acid requirement. There follows a full set of reactions including these processes, with two distinct sets of pool-holding sites.

Concentrating mechanism.

$$A + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \overline{AE}$$

$$R + \overline{AE} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \overline{AR} + E$$

$$R' + \overline{AE} \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} \overline{AR}' + E$$

Synthesis and utilization.

$$\overline{AE} \xrightarrow{\alpha} E + \text{Protein}$$

$$\overline{XA} + E \xrightarrow{k_5} \overline{AE}$$

The last reaction represents a possible scheme for the cell's control of the rate of amino acid

synthesis. It is proposed that amino acid is synthesized and remains associated with the final synthetic enzyme site, forming the complex, $\overline{XA}$. When a free carrier collides with this complex, the amino acid is taken up by the carrier. Essentially instantly another internally synthesized amino acid is formed, so that the concentration of $\overline{XA}$ is constant. The rate of flow of amino acid from the synthetic site is therefore $k_5\overline{XA}E$, which is written as βE for simplicity. Thus, in this model the concentrations of carrier complex and free carrier control not only the rate of incorporation of amino acid and the quantity of pool but also the rate of internal synthesis of amino acid.

The total quantities of carrier and pool holding sites are both limited.

$$E + \overline{AE} = z \tag{1}$$

$$R + \overline{AR} = \pi \quad \text{and} \quad R' + \overline{AR}' = \pi' \tag{2}$$

The rates of change of the carrier complex and site complex (pool) are as follows,

$$\frac{d\overline{AE}}{dt} = k_1 EA - k_2\overline{AE} + k_4 E\overline{AR} - k_3 R\overline{AE} + k_4' E\overline{AR}' - k_3' R'\overline{AE} + \beta E - \alpha \tag{3}$$

$$\frac{d\overline{AR}}{dt} = k_3 R\overline{AE} - k_4\overline{AR}E \tag{4}$$

$$\frac{d\overline{AR}'}{dt} = k_3' R'\overline{AE} - k_4'\overline{AR}'E \tag{5}$$

and their sum is

$$\frac{d\overline{AE}}{dt} + \frac{d\overline{AR}}{dt} + \frac{d\overline{AR}'}{dt} = k_1 EA - k_2\overline{AE} + \beta E - \alpha \tag{6}$$

When a steady pool exists, equations 3, 4, 5, and 6 are equal to zero. Under these conditions, the fraction of the carrier that is free may be calculated from equation 6 by using equation 1.

$$\frac{E}{z} = \frac{k_2 + \alpha/z}{k_1 A + k_2 + \beta} \tag{7}$$

The rate of synthesis of amino acid is

$$\beta E = \beta \frac{\alpha + k_2 z}{k_1 A + k_2 + \beta} \tag{8}$$

The net rate of flow of amino acid from the environment into the cell is

$$k_1 EA - k_2\overline{AE} = \alpha \frac{k_1 A + k_2 - \beta k_2 z/\alpha}{k_1 A + k_2 + \beta} \tag{9}$$

The steady pool size may be calculated by setting equations 4 and 5 equal to zero and using equations 1, 2, and 7.

$$\overline{AR} + \overline{AR}' = \frac{\pi}{1 + \dfrac{k_4(k_2 + \alpha/z)}{k_3(k_1 A + \beta - \alpha/z)}} + \frac{\pi'}{1 + \dfrac{k_4'(k_2 + \alpha/z)}{k_3'(k_1 A + \beta - \alpha/z)}} \tag{10}$$

Equation 10 has the form of the sum of two adsorption isotherms in the higher concentration range, but automatically includes the native pool at the very lowest concentrations. Numerically it is essentially indistinguishable from a simple sum of the native pool and two classical adsorption isotherms.

The native pool formed when no supplemental amino acid has been added to the culture may also be calculated. Since all of the amino acid is internally synthesized in this case, $\beta E_0 = \alpha$. Setting equations 4 and 5 equal to zero and noting that, because the native pool is small, $R \simeq \pi$ and $R' \simeq \pi'$, we obtain

$$\overline{AR}_0 + \overline{AR}_0' \simeq \frac{k_3\pi}{k_4}\left(\frac{z\beta}{\alpha} - 1\right) + \frac{k_3'\pi'}{k_4'}\left(\frac{z\beta}{\alpha} - 1\right) \tag{11}$$

Also, since in this case $k_1 A_0 E = k_2\overline{AE}$, the corresponding external concentration is

$$A_0 = \frac{k_2}{k_1}\left(\frac{z\beta}{\alpha} - 1\right) \tag{12}$$

The initial total rate of incorporation of amino acid from the medium at the instant the amino acid is added may be calculated by using the steady-state approximation. The rate of change of the amount of carrier complex, $\overline{AE}$, is taken to be zero, after an extremely short period during which it rises to its slowly changing value. A numerical check with the constants listed in Table 7 has shown this approximation to be very good. Again, because of the small size of the native pool, we may put $R = \pi$ and $R' = \pi'$. Equation 3 then becomes

$$k_1 EA - (k_3\pi + k_3'\pi')\overline{AE} + (k_4\overline{AR}_0 + k_4'\overline{AR}_0')E + \beta E - \alpha \simeq 0$$

TABLE 7. *Numerical evaluation of constants for the carrier model: concentration of proline by E. coli*

Parameter	Symbol	Numerical value
Data utilized:		
1. Quantity of sites, first component	π	8.0×10^{-2} mole/liter
2. A_k[a] for sites, first component		1.0×10^{-4} mole/liter
3. Quantity of sites, second component	π'	4.3×10^{-3} mole/liter
4. A_k for sites, second component		1.0×10^{-6} mole/liter
5. Maximum total rate of incorporation		6.0×10^{-5} mole/liter $\times$ sec
6. A_k for total rate of incorporation		1.5×10^{-6} mole/liter
7. Rate of utilization for protein synthesis		1.0×10^{-5} mole/liter $\times$ sec
8. Native pool		2.0×10^{-4} mole/liter
9. Native concentration ratio		2.5×10^{4}
Derived constants:		
Arbitrarily chosen	z	1.0×10^{-5} mole/liter
From native pool (8)[b]	β	1.16 sec^{-1}
From A_k for rate (6)	k_1	4.80×10^{6} liters/mole $\times$ sec
From native concentration ratio (9)	k_2	0.25 sec^{-1}
From maximum rate (5)	$k_3 = k_3'$	59.3 liters/mole $\times$ sec
From A_k, first component (2)	k_4	2.26×10^{4} liters/mole $\times$ sec
From A_k, second component (4)	k_4'	2.33×10^{2} liters/mole $\times$ sec

[a] Represents the external concentration at which each parameter reaches one-half of its maximum value.

[b] The experimental parameter listed is dominant in determining the constant, although the value is, of course, influenced by other parameters. Numbers in parentheses refer to the equations.

Using equation 1 and substituting $\overline{AR}_0$ and $\overline{AR}_0'$, we may solve for E and then write the initial total rate of incorporation from the environment.

$$k_1 AE \simeq \frac{k_1 A(k_3\pi z + k_3'\pi' z + \alpha + k_2 z)}{k_1 A + \frac{z\beta}{\alpha}(k_3\pi + k_3'\pi') + \beta + k_2} \qquad (13)$$

In the experiments designed to measure the small leak rate, $k_2\overline{AE}$, protein synthesis was inhibited, the pool was fairly large, and the external concentration was very low. Examination of the equations and numerical analysis show that under these conditions the amount of free carrier will be very small, and therefore the loss rate will be simply $k_2 z$.

E. Evaluation of the Rate Constants of the Carrier Model

Analysis of the experimental data. Figure 31 presents the best available data for the numerical evaluation of the constants of the carrier model for proline in *E. coli*. The results presented in this figure are principally those shown in Fig. 20, converted to chemical units. However, a more precise method for the calculation of the pool size has been utilized which influences somewhat the values at low concentrations. In addition, an experiment at a very low concentration, which was carried out simultaneously, has been included.

When radioactive amino acid is added at low concentrations, it is diluted by internally synthesized amino acid. Thus, the radioactivity of the pool is not a direct measure of the pool size. However, the pool size may be calculated precisely if it is assumed that the pool is the source of amino acid utilized for protein synthesis and that the unlabeled, internally synthesized and the labeled amino acid are completely mixed in the pool. The rate of incorporation of labeled amino acid ($\overline{AR}^*$) into protein is

$$\frac{dp^*}{dt} = \frac{\overline{AR}^*}{AR}\alpha$$

Thus

$$\overline{AR} = \frac{\alpha\overline{AR}^*}{dp^*/dt}$$

Here $\overline{AR}^*$ and dp^*/dt are the measured radioactivity of the pool and rate of incorporation of radioactivity into protein. α, the total rate of utilization for protein synthesis, was evaluated

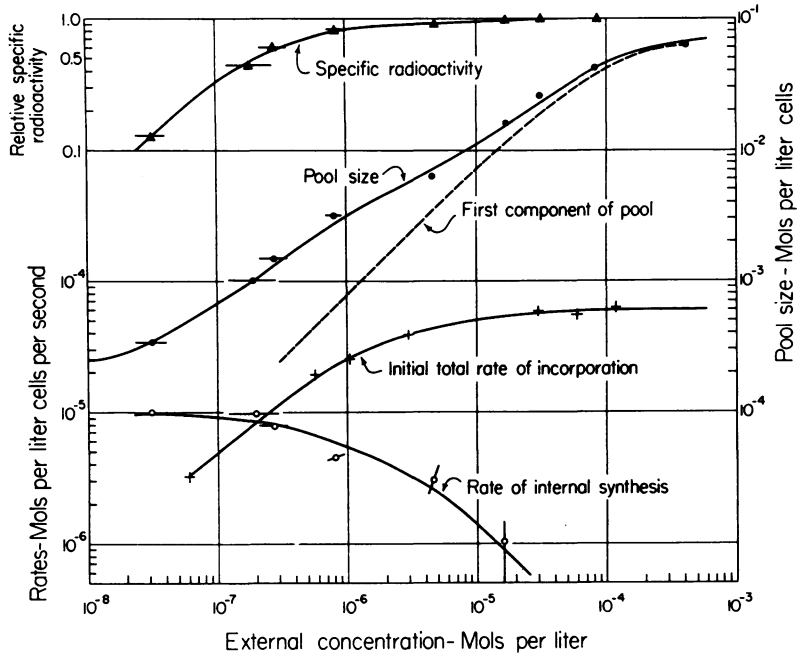

FIG. 31. *Proline pool formation in E. coli at 25 C; log-log plot. The experimental points are derived from a set of eight simultaneous measurements of the time course of incorporation of radioactive proline, except for the point at 4.2 × 10^{-4} M, which is the average of several measurements of the saturation pool size.* ▲, *Ratio of specific radioactivity of the pool (at the time it reached its maximum value) to that of the added radioactive proline.* ●, *Maximum pool size reached versus concentration present at that time.* – – –, *Prediction of the carrier model for the size of the major component of the pool alone.* +, *Initial total rate of incorporation of external proline.* ○, *Calculated average rate of internal synthesis of proline during the time required for the pool to reach its maximum value.*

from several experiments at high external concentration where internal synthesis was completely suppressed. For Fig. 31, $\overline{AR}$ was evaluated by means of this equation at the time the rate of change of the pool was zero, for each of the eight experiments. There remains an uncertainty in the external concentration present at this time, since internally synthesized amino acid may appear in the environment by exchange with the radioactive amino acid added. As a correction could not be made without a knowledge of the exchange rate, the probable limits of error due to exchange are shown by the horizontal bar through each point.

The curve drawn through the experimental points for the pool size is the sum of two adsorption isotherms and the native pool. This is the curve predicted by the carrier model (equation 10) for the constants listed in Table 7.

The next stage in the analysis is to calculate the rate of internal synthesis of amino acid. Both the radioactivity and the size of the pool are known at the time the pool reaches its maximum value, and thus the specific radioactivity of the pool may be calculated. The upper curve on Fig. 31 shows the ratio of the specific radioactivity of the pool (at the time it reached its maximum value) to that of the originally added amino acid. The dilution observed at the lower concentrations arises from internal synthesis during the time required to build the pool and from the "native" pool initially present. (An examination of the ratio of internal to external concentration in the experiment at the lowest concentration shows that the external amino acid originally present in the culture makes but a small contribution to the dilution.)

A precise calculation of the average rate of internal synthesis during the time required to build the pool would require a knowledge of the exchange rate, since a certain amount of the internally synthesized amino acid appears in the environment by exchange. However, the calculation is possible for the case of zero exchange

and the case for which the exchange rate is so great that the internal and external specific radioactivities are always equal. By such calculations one can place limits on the rate of internal synthesis. The probable outside limits are plotted at their appropriate external concentrations and joined by solid bars.

For the two experiments at the lowest concentrations, the rate of synthesis was taken to be equal to the requirement for protein synthesis, and thus the native pool could be calculated. For each of these experiments the result was $2.0 \pm 1.0 \times 10^{-4}$ mole per liter of cells, and this value was used in evaluating the four cases at higher concentrations.

The final step in the analysis of the experiments is to calculate the initial rate of incorporation of exogenous amino acid. For this purpose the radioactivity remaining in the environment was plotted on semilogarithmic paper. For the first four cases (below 10^{-5} M external concentration), a straight line fitted the data accurately for times extending almost out to the time when the maximum pool size was reached. Thus an accurate value of the time constant (T) could be obtained, and the initial rate of incorporation, $-dA/dt$, was easily calculated from the relation

$$\frac{1}{T} = -\frac{1}{A^*}\frac{dA^*}{dt} = -\frac{1}{A}\frac{dA}{dt}$$

For the experiments at the four higher concentrations, the less accurate but essentially equivalent methods mentioned in Part II-I were utilized.

It is interesting from the point of view of the model that the external concentration decays exponentially, since

$$\frac{1}{\rho T} = -\frac{1}{\rho A}\frac{dA}{dt} = k_1 E - \frac{k_2 \overline{AE}}{A}$$

The small rate of loss observed when the external concentration was strongly reduced, and the high ratio of internal to external concentration observed in the experiment at the lowest concentration, both show that the second term, $k_2\overline{AE}/A$, is negligible. Therefore, in the four experiments below 10^{-5} M, E, the amount of free carrier, is nearly constant with time. In the three experiments at the lowest concentrations, E in fact increases slightly as the maximum pool is approached. In the four experiments at the higher concentrations, E decreases as the maximum pool is approached.

Numerical evaluation of constants. The curve drawn through the experimental points for the pool size is the sum of the native pool and two adsorption isotherms with the constants shown in Table 7 (items 1, 2, 3, and 4). The first pair of constants, π and π', are simply the saturation values of the two components.

The total quantity of carriers, z, has no influence on the predictions of the model as long as it is small. It has therefore been arbitrarily chosen to be 5% of the native pool or 6,000 carriers per cell. All of the derived constants are inversely proportional to z except π and π'.

Equations 10, 11, 12, and 13 each include several of the constants to be evaluated. The resulting set of simultaneous equations was solved by successive approximations. An approximate form was written down for each of the equations, eliminating unimportant terms. These equations were then numerically solved, and the resulting approximate constants were substituted in the complete equations. Since each of the experimental parameters dominantly affects a particular constant, it is a simple matter to adjust the constants (in proper sequence) until an accurate fit is obtained. Actually the system converges in one cycle.

There is no experimental evidence available which allows the rates of formation of the two components of the pool to be evaluated separately. Therefore, k_3 and k_3' were arbitrarily chosen to be equal.

With the exception of the native pool and native concentration ratio, (that is, the ratio of pool per liter of cells to external amino acid per liter of medium), the experimental parameters are known to be better than 20%. The determination of the native pool has been discussed above. The native concentration ratio was estimated from the experiment at the lowest concentration shown on Fig. 31. This experiment cannot be interpreted unambiguously because of the unknown rate of exchange between the pool (which is continuously being diluted by internal synthesis) and the external amino acid. The uncertainty in these numbers probably is a factor of 2 either way. The measurement of the rate of loss shown in Fig. 23 implies a lower value of k_2, but this was performed with a different strain of *E. coli*.

The measured values of the rate of internal synthesis of proline have not been used in the

evaluation of the constants. The lowest curve shown on Fig. 31 was calculated from the carrier model by using the constants shown in Table 7. This calculation is for the average rate of internal synthesis during the period of pool formation for these particular experiments. For steady pool and constant external concentration, the model predicts a similarly shaped curve, which falls to half-value at 3.1×10^{-7} M.

The agreement with the experimental points probably is within the experimental error. This demonstrates the adequacy of the carrier model in terms of our present experimental knowledge.

It is interesting to note that if one calculates the size of the two components as indicated above for the exchange experiment shown in Fig. 18, then the exchange rate of each component is proportional to its size with a single proportionality constant.

V. Acknowledgment

In writing this article we have used freely the experiments and concepts of our colleagues at the Department of Terrestrial Magnetism, especially R. B. Roberts, D. B. Cowie, and E. T. Bolton. They have also liberally given guidance and advice. We are much indebted to the wife (B. H. B.) of one of us for assistance with the typing and editing.

VI. Literature Cited

1. Aronson, A. I., E. T. Bolton, R. J. Britten, D. B. Cowie, J. D. Duerksen, B. J. McCarthy, K. McQuillen, and R. B. Roberts. 1960. Carnegie Institution of Washington Yearbook **59**: 229–279.
2. Bolton, E. T., R. J. Britten, D. B. Cowie, E. H. Creaser, and R. B. Roberts. 1956. Carnegie Institution of Washington Yearbook **55**:110–148.
3. Bolton, E. T., R. J. Britten, D. B. Cowie, J. J. Leahy, F. T. McClure, and R. B. Roberts. 1957. Carnegie Institution of Washington Yearbook **56**:118–142.
4. Bolton, E. T., R. J. Britten, D. B. Cowie, and R. B. Roberts. 1958. Carnegie Institution of Washington Yearbook **57**:127–162.
5. Britten, R. J., and F. T. McClure. 1959. The osmotic properties of the metabolic pool of *E. coli*. Proc. First National Biophysics Conference, p. 414–423. Yale University Press, New Haven, Conn.
6. Britten, R. J., R. B. Roberts, and E. F. French. 1955. Amino acid adsorption and protein synthesis. Proc. Natl. Acad. Sci. U. S. **41**:863–870.
7. Cohen, G. N., and H. V. Rickenberg. 1956. Concentration spécifique réversible des amino acids chez *Escherichia coli*. Ann. inst. Pasteur **91**:693–720.
8. Cohen, G. N., and J. Monod. 1957. Bacterial permeases. Bacteriol. Rev. **21**:169–194.
9. Demerec, M., and J. Hanson. 1951. Mutagenic action of manganous chloride. Cold Spring Harbor Symposia Quant. Biol. **16**:215–228.
10. Hendler, R. W. 1958. Possible involvement of lipids in protein synthesis. Science **128**:143–144.
11. McCarthy, B. J., and R. J. Britten. 1962. The synthesis of ribosomes in *E. coli*. I. The incorporation of C^{14}-uracil into the metabolic pool and RNA. Biophys. J. **2**:35–47.
12. McQuillen, K., R. B. Roberts, and R. J. Britten. 1959. Synthesis of nascent protein by ribosomes in *E. coli*. Proc. Natl. Acad. Sci. U. S. **45**:1437–1447.
13. Roberts, R. B., and E. Aldous. 1951. Manganese metabolism of *E. coli* as related to its mutagenic action. Cold Spring Harbor Symposia Quant. Biol. **16**:229–231.
14. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1955. Studies of biosynthesis in *E. coli*. Carnegie Inst. Wash. Publ. No. 607.